Journal of Tropical Microbiology and Biotechnology

The International Journal for Microbiologists and Biotechnologists working on Issues Relevant to the Tropics

Volume 3 Number 2

(December 2007)

J. Trop. Microbiol. Biotechnol. 2007, Vol. 3 No. 2

1

Contents

Editorial Comment

Welcome to this issue of *Journal of Tropical Microbiology and Biotechnology (JTMB 3(2)*. Our journal (formerly *Journal of Tropical Microbiology*) is one of 220 journals listed by African Journals Online (AJOL, http://www.ajol.info/) and is also indexed by CABI and Chemical Abstracts. Since the first issue of JTMB published in 2005, more people have submitted their articles for publication in the journal. In this issue of the journal, the first paper focuses on the plant pathogen *Phaeoisariopsis griseola* and angular leaf spot development in common bean**.** Crop pests continue to hamper food security in the region and it is important to continue to study all dimensions of pests afflicting important crops like the common bean.. The 2nd paper looks the potential use of *Klebsiella oxytoca* '10mkr7', a bacterial strain to stimulate *Striga* suicidal germination in *Zea mays.* Again, Maize is an important crop in the region and *Striga hamonthica* continues to be a challenge to maize production. Attempts at biological control of the weed are an important step towards eventual management and control of the weed. The 3rd paper looks heavy metal tolerance and antibiotic resistance profiles of gram-negative bacteria isolated from Lake Victoria, Kenya. Lake Victoria is an important resource in Eastern Africa and human activities continue to impact on the flora and fauna in the lake, which is likely to affect the users of that important ecosystem. Research into this fragile ecosystem should help in its sustainable utilization. The 4th paper looks at the screening of local *Bacillus thuringiensis* isolates for toxicity to C*hilo partellus, Sesamia calamistis* and *Busseola fusca* in Kenya. The three insects are important pests of maize and contribute to reduced crop production. Local isolates of *B. thuringiensis* are necessary for use in biological control of the pests under local conditions, but can also serve as a source of novel genes for use in modern biotechnology. The 5th paper also looks at the development of a Latex Agglutination Test (LAT) for Capripoxyirus. Capripoxyviruses cause sheeppox, goatpox and lumpyskin diasease, and the LAT appears to be a useful tool for rapid diagnosis. The 6th paper on Lead (Pb) uptake (mediated by abuscular myorrhiza) from Soil and Pb Deposit on Cowpea plant takes us back to the recurrent them of the impact of human activities (in this case lead pollution) on food security. This is especially important as it give insight into the potential impact of lead pollution from vehicles on urban agriculture. The final paper looks at the use of fungi to control the root-knot nematode *Meloidogyne incognita* an important plant pest responsible for severe crop damage in tropical subsistence agriculture. Again control of crop pests is important in ensuring food security and suitable biological agents can contribute to safe control measures that do not harm the environment. We would like to thank our readers, authors and reviewers for their continued support and enthusiasm for the Journal.

Hamadi Boga
Assistant-Editor in Chief
hamadiboga@yahoo.com

Variation in aggressiveness of *Phaeoisariopsis griseola* and angular leaf spot development in common bean

Wagara I. N.[1*], Mwang'ombe A. W.[2], Kimenju J. W.[2] and Buruchara R. A.[3]

[1] Department of Biological Sciences, Egerton University, P.O BOX 536, Njoro, Kenya
[2]Department of Plant Science and Crop Protection, University of Nairobi, P.O BOX 30197, Nairobi, Kenya
[3]Centro Internacional de Agricultura Tropical (CIAT), Kawanda Agricultural Research Institute, P.O BOX 6247, Kampala, Uganda.
*Author for correspondence (E-mail: inwagara@yahoo.com)

ABSTRACT

Aggressiveness of fifteen isolates of *Phaeoisariopsis griseola* and variations in angular leaf spot symptom development in common bean were studied. The isolates were selected based on their virulence and genetic differences and represented Andean, Afro-Andean and Mesoamerican groups of *P. griseola*. Aggressiveness was determined based on length of incubation period, rate of lesion expansion, lesion size and density, rate of sporulation, disease severity and area under disease progress curve (AUDPC). There was a wide variation in aggressiveness and the type of symptoms induced by the different isolates of the pathogen. Incubation period varied significantly (P < 0.05) among isolates and ranged from six to 15 days. Mesoamerican isolates had significantly shorter incubation period than Andean and Afro-Andean isolates. Disease severity, AUDPC and rate of sporulation differed significantly (P < 0.05) among the isolates. Lesions induced by the different isolates varied significantly in size, density and rate of expansion. Mesoamerican isolates induced significantly more lesions than Andean and Afro-Andean isolates. Disease severity was negatively correlated to incubation period and positively correlated to lesion density, lesion size and rate of sporulation. The significant correlations between disease severity, AUDPC, incubation period, lesion density and rate of sporulation indicate that all these parameters are important measures of aggressiveness in *P. griseola*. The significant variations in aggressiveness between isolates, virulence and genetically defined groups of *P. griseola* indicate that this parameter can be used to characterise isolates of the pathogen. Symptoms induced by isolates of *P. griseola* in different bean cultivars varied extensively in size, shape and time of appearance. These variations could, however, not be attributed to any particular cultivar or isolate and can, therefore, not be used to characterise the pathogen.

Key words: Disease severity, incubation period, sporulation, symptom, virulence group

INTRODUCTION

Amongst the legumes grown in Kenya, common bean (*Phaseolus vulgaris* L.) is ranked as the most popular in both production and utilisation. Bean is grown primarily by small-scale farmers who have limited resources and usually produce the crop under adverse conditions such as low input use, marginal lands and intercropping with competitive crops (Wortmann *et al.*, 1998). Angular leaf spot (ALS), caused by the imperfect fungus *Phaeoisariopsis griseola* (Sacc.) Ferraris, is a major constraint to bean production in tropical and subtropical countries (Saettler, 1991, Liebenberg and Pretorius, 1997, Wortmann *et al.*, 1998). In Africa, particularly in Kenya, Malawi, Ethiopia, Uganda, Tanzania and the Great Lakes Region, where beans constitute the most important source of dietary protein, ALS is considered the number one constraint to bean production with annual losses estimated at 374,800 tonnes (Wortmann *et al.*, 1998). The disease affects foliage and pods throughout the growing season and is particularly destructive in areas where warm, moist conditions are accompanied by abundant inoculum from infected plant residues and contaminated seed (Saettler, 1991). Severe yield losses of up to 80% have been reported under favourable environment

(Correa-Victoria, 1988; Liebenberg and Pretorius, 1997; Stenglein *et al.* 2003). Most of the crop losses result from premature defoliation that mainly occurs starting from the flowering and beginning of pod-filling stage (Mwang`ombe *et al.*, 1994).

Studies based on isozymes, differential cultivars, random amplified polymorphic DNA (RAPD) markers, microsatellites and amplified fragment length polymorphism (AFLP) have revealed high pathogenic and genetic variations in *P. griseola* (Boshoff *et al.*, 1996, Wagara, 1996, Chacon *et al.*, 1997, Pastor-Corrales *et al.*, 1998, Busogoro *et al.*, 1999, Guzman *et al.*, 1999, Mahuku *et al.,* 2002b, Wagara *et al.*, 2004). Isolates of the pathogen have been divided into Andean and Mesoamerican groups, which correspond to the two common-bean gene pools (Guzman *et al.,* 1995; CIAT, 1996; Pastor-Corrales *et al.*, 1998). The Andean group consists of *P. griseola* isolates that exclusively infect Andean genotypes whereas the Mesoamerican isolates, although more virulent and aggressive on Mesoamerican bean genotypes, also infect Andean varieties (Pastor-Corrales *et al.,* 1998, Mahuku *et al.*, 2002a). Pathogen characterisation carried out in Africa indicates occurrence of a third virulence group of *P. griseola* isolates: the Afro-Andean (CIAT, 1997; Mahuku *et al.*, 2002a). These

isolates infect Andean differential cultivars plus one or two Mesoamerican genotypes.

Considerable variations occur in the morphology of *P. griseola* isolates and angular leaf spot development. The fungus produces synnemata that are 20-40μm wide (Liebenberg and Pretorius, 1997). The number of conidiophores to a synnema and the size of synnema show considerable variation. The number of synnemata per lesion range from 5 - 52 and the synnemal conidiophores range from 15 - 106 (Wagara, 1996). The incubation period is reported to vary from 12-14 days (Anon., 1978), 5-16 days (Buruchara, 1983), 10-12 days (Correa and Saettler, 1987) and 6-15 days (Wagara *et al.*, 1999). Angular leaf spot symptoms can appear on leaves within six days after inoculation as dark grey to brown lesions (Wagara, 1996), but do not become prevalent until after the late flowering or early pod-set stage (Barros *et al.*, 1958). On the primary leaves, the lesions tend to be circular but on the trifoliates, the spots are delimited by veins and veinlets, giving them a characteristic angular shape (Saettler, 1991; Wagara, 1996; Liebenberg and Pretorius, 1997). Under severe infection, lesions coalesce and premature defoliation occurs. On pods, symptoms appear as large, circular to elliptical reddish-brown lesions with a slightly darker perimeter. Infection may spread to the underlying seeds, which then become discoloured and malformed. On stems and petioles, lesions are dark brown and elongate (Saettler, 1991).

Although a lot of variations have been noted in the development of angular leaf spot and the type of symptoms induced by different isolates of *P. griseola* (Liebenberg and Pretorius, 1997; Stenglein *et al.*, 2003), no comprehensive study has been done to investigate these differences and their relationships. Such information would be important in determining whether these variations are related to aggressiveness and/or pathotype differences of the pathogen. A lot of discrepancies exist in determining aggressiveness of a pathogen. Correa-Victoria (1988) measured aggressiveness of *P. griseola* isolate on a cultivar as the 'number of days after inoculation until 20% disease severity was evident', which he called incubation period. He proposed that this be replaced by the number of days after inoculation until 50% of lesions contained spores. He also measured sporulation capacity, expressed as the number of spores produced by mm^2 of lesion. According to Andrivon (1993), aggressiveness refers to the severity of disease induced by an

isolate for a particular host-pathogen interaction. There is need to investigate and determine the aspects of disease development and/or pathogen reactions that can be used as true measures of aggressiveness in *P. griseola* isolates. The objectives of this study were, therefore, to evaluate the variations in symptoms and aggressiveness of *P. griseola* isolates and determine the possible application of these parameters in pathogen characterisation.

Materials and Methods
Choice of *P. griseola* isolates and evaluation for angular leaf spot development

Fifteen isolates of *P. griseola* representing 15 races of the pathogen were used in this study (Table 1). The isolates were selected based on their virulence reactions on 12 bean differential cultivars and characterisation based on AFLP fingerprints (Wagara *et al.*, 2004). The isolates were representative of Andean, Afro-Andean and Mesoamerican groups of the pathogen. Four of the isolates were Andean (eb2, mk12, eb11, eb15), six were Afro-Andean (kk16, tt14, kk13, tt8, kb17, tt6) and five were Mesoamerican (tt17, mk19, kb12, kb6, tt16). Three week-old seedlings of Rosecoco GLP-2 were spray-inoculated with inoculum from 14 day-old cultures of *P. griseola* at a concentration of 2×10^4 conidia ml^{-1} until runoff. Inoculated plants were incubated in a growth chamber at 24°C and relative humidity of >90%. The experiment was replicated six times. Plants were monitored daily for ALS development. Aggressiveness was determined based on length of incubation period, rate of lesion expansion, lesion size and density, rate of *in vivo* and *in vitro* sporulation, disease severity, area under disease progress curve (AUDPC), number of conidiophores per synnema and number of synnemata per lesion. Length of incubation period was measured as the number of days from inoculation to appearance of the first symptoms. For each isolate-cultivar interaction, ten lesions were randomly selected and measured at two-day intervals for two weeks to determine their rate of expansion. Seventeen days after inoculation, 10 leaves were selected and the number of lesions in a 4 cm^2 area counted to determine lesion density. Disease severity was determined four times after every two days starting from the time of symptom appearance, as the percent leaf area infected. Area under disease progress curve was calculated using the formula of Shaner and Finney (1977):

$$AUDPC = \sum_{i=1}^{n-1} \left[(Y_{i+1} + Y_i)/2 \right] \left[X_{i+1} - X_i \right]$$

Where Y_i = disease severity at i^{th} observation, X_i = number of days after inoculation at the ith observation, and n = total number of observations.

Table 1. Origin, race, virulence and genetic groups of *Phaeoisariopsis griseola* isolates used to study variations in aggressiveness of the angular leaf spot pathogen

Isolate	District of origin	Race	Virulence group	AFLP group
Eb2	Embu	6-0	Andean	I
Mk12	Machakos	26-0	Andean	I
Eb11	Embu	34-0	Andean	II
Eb15	Embu	46-0	Andean	II
Kk16	Kakamega	22-32	Afro-Andean	I
Tt14	Taita Taveta	30-1	Afro-Andean	II
Kk13	Kakamega	30-4	Afro-Andean	II
Tt8	Taita Taveta	46-2	Afro-Andean	II
Kb17	Kiambu	56-2	Afro-Andean	II
Tt6	Taita Taveta	63-32	Afro-Andean	II
Tt17	Taita Taveta	31-7	Mesoamerican	II
Mk19	Machakos	59-54	Mesoamerican	I
Kb12	Kiambu	62-7	Mesoamerican	I
Kb6	Kiambu	63-55	Mesoamerican	I
Tt16	Taita Taveta	63-63	Mesoamerican	I

Determination of rate of *in vivo* and *in vitro* sporulation

Rate of *in vivo* sporulation was determined by cutting-out twenty pieces (3 mm² each) of randomly selected sporulating lesions and agitating them vigorously for about two minutes in 2 ml of sterile water. The solution was filtered through a double layer of cheesecloth to remove plant debris and mycelial fragments. The experiment was replicated thrice. Spore concentration was determined by making 10 haemocytometer counts from each replicate and taking their average. To determine the number of conidiophores per synnema and synnemata density, lesions were cleared using the method described by Bruzzese and Hasan (1983). Pieces of leaf tissue (1 cm²) containing sporulating lesions were cut out and immersed in the clearing and staining solution (2 ml/cm² of tissue) in stoppered glass vials for 48 hours at room temperature. The tissues were then removed and placed in concentrated chloral hydrate solution (2.5 g/ml water) for 48 hours. They were rinsed in distilled water, mounted in clear lactophenol and observed under the light microscope. Ten lesions were randomly selected and the number of synnemata per 2.5 mm² determined. Five synnemata were selected per lesion and the number of conidiophores per synnema determined.

To determine the rate of *in vitro* sporulation, cultures of *P. griseola* were prepared by inoculating bean leaf dextrose agar (BLDA) plates (Wagara, 1996) with 250 μl of a spore suspension containing 2×10^4 conidia ml⁻¹. The plates were swirled to distribute the spores evenly and incubated in a non-illuminated incubator at 24°C for 14 days. Five millilitres of sterile distilled water were then added to the plates and the culture surface gently scrapped with a sterile glass rod. The spore suspension was filtered through two layers of cheesecloth and the spore concentration determined as above. Three replicates were used and the experiment was repeated thrice.

Evaluation of angular leaf spot symptoms induced by isolates of *P. griseola*

Fifteen bean cultivars of various seed sizes and growth habits were used to study the variations in symptoms induced by *P. griseola* isolates. They included the twelve angular leaf spot bean differential cultivars (Don Timoteo, G11796, Bolon Bayo, Montcalm, Amendoim, G5686, PAN72, G2858, Flor de Mayo, Mexico 54, BAT332 and Cornell 49242). In addition, three local bean cultivars (Rosecoco GLP-2, Mwitemania GLP-X.92 and No.B) were also included. Three-week old seedlings of the

cultivars were separately inoculated with different isolates of *P. griseola* and evaluated for development of angular leaf spot symptoms. Inoculum preparation, plant inoculation and incubation were done as described above. Variations in time of symptom appearance and size and shape of lesions induced by the different isolates were noted.

Data analysis

Data on disease severity was transformed using arcsine transformation whereas data on *in vivo* and *in vitro* sporulation, lesion density, number of conidiophores per synnema and synnemata density were transformed using logarithmic transformations (Gomez and Gomez, 1984). The data was then analysed by Analysis of Variance (ANOVA) using GenStat Release 6.1 (GenStat Procedure Library Release PL14). Separation of means was done using least significant difference (LSD). Correlation coefficients were derived for the different parameters, namely disease severity, AUDPC, incubation period, lesion size, lesion density, rate of lesion expansion, *in vivo* and *in vitro* sporulation.

RESULTS

Variation in aggressiveness of *P. griseola* isolates

P. griseola isolates showed wide variations in their levels of aggressiveness. Incubation period varied significantly (P<0.05) among the isolates and ranged from six to 15 days (Table 2). On the average, Mesoamerican isolates had a significantly shorter incubation period (8 days) than Afro-Andean (11 days) and Andean isolates (13 days) (Table 3). Mesoamerican isolates tt17 and mk19 had the shortest incubation period of six days, whereas Andean isolate eb2 had a significantly longer incubation period of 15 days. The isolates caused varying levels of disease, ranging from 6.7% severity induced by Andean isolate eb11 to 40.7% induced by Mesoamerican isolate tt16. Mesoamerican isolates induced significantly (P<0.05) higher disease severity (average of 33.5%) than Andean and Afro-Andean isolates (19.5 and 22.5%, respectively) (Table 3). When the isolates were grouped into genetically different groups based on their AFLP fingerprints (Table 1), AFLP group I (corresponding to Mesoamerican isolates)

induced significantly (P<0.05) higher levels of disease severity than AFLP group II (corresponding to Andean and Afro-Andean isolates) (Table 4). On the average, they also had a shorter incubation period (nine days as compared to 11 days for AFLP group II).

Lesions induced by the different isolates varied significantly (P<0.05) in size, density and rate of expansion (Table 2). The largest lesions recorded in this study (8.4 mm^2) were induced by Mesoamerican isolate tt16 whereas Afro-Andean isolate tt14 induced the smallest lesions (1.1 mm^2). Mesoamerican isolates induced significantly more lesions per unit area than the Andean and Afro-Andean isolates. Isolate mk12 induced the highest number of lesions (8.8 lesions/cm^2) whereas isolate eb11 induced the least (one lesion/cm^2) (Table 2). The rate of lesion expansion also differed significantly (P<0.05) among the isolates. Lesions induced by isolate kb6 had the highest rate of expansion (0.92mm^2/day) as compared to the low expansion rate (0.22 mm^2/day) of lesions induced by isolate tt17.

All the isolates sporulated significantly (P<0.05) more heavily on the host than in culture and there was a significant difference among the isolates in their rate of *in vivo* and *in vitro* sporulation (Table 2). Mesoamerican isolates sporulated significantly (P<0.05) more heavily than Andean and Afro-Andean. Mesoamerican isolate kb6 had the highest sporulation rate whereas Andean isolate eb11 had the lowest. Afro-Andean isolates had the highest rate of *in vitro* sporulation whereas the highest rate of *in vivo* sporulation was recorded with Mesoamerican isolates (Table 3). Andean isolates, on the other hand, had the lowest sporulation rates, both *in vivo* and *in vitro*. Mesoamerican isolates had a significantly (P<0.05) higher lesion density, synnemata per lesion and conidiophores per synnema when compared to Andean and Afro-Andean. Mesoamerican isolate tt16 had the highest synnemata density per lesion (18.6 synnemata/lesion) whereas Andean isolate eb11 had the lowest (6.1 synnemata/lesion). Isolate eb11 also had the lowest number of conidiophores per synnema.

Table 2. Variation in ALS severity, incubation period, lesion development, AUDPC and sporulation of *Phaeoisariopsis griseola* isolates

Isolate	Severity (%)	AUDPC	Incubation period (days)	Lesion size (mm^2)	Lesion density[a]	Lesion expansion (mm^2/day)	*Sporulation *in vivo*	*Sporulation *in vitro*	Synnemata per lesion	Conidiophore per synnema
Eb2	26.3	88.2	15	4.6	2.2	0.6	44.7	11.8	13.6	26.4
Mk12	36	181.5	8	2.6	8.8	0.4	55	13.5	14.4	21.5
Eb11	6.7	31.1	13	3.6	1.0	0.4	26.7	4.8	6.1	16.4
Eb15	15.7	68.7	14	7.1	2.2	0.3	44	9.8	10.1	16.9
Kk16	8	33.2	11	1.9	1.7	0.3	43.3	8.5	8.4	17
Tt14	27.7	152.5	7	1.1	2.9	0.5	63.3	25.7	12.8	25.2
Kk13	7.8	87	12	5	7.7	0.6	63.8	24.5	17.6	29.1
Tt8	21.3	87.3	11	4.1	2.5	0.4	33.3	6.6	8.7	17.1
Kb17	20	117	13	6.2	1.4	0.8	64.7	31.2	12	31.4
Tt6	35.7	170	12	4.1	4.5	0.5	36	7.4	8.3	16.7
Tt17	24	133.5	6	1.7	6.1	0.2	62.7	21.8	13.8	21.1
Mk19	35.3	127	6	2.3	6.6	0.3	62	18	11.4	21.9
Kb12	26.7	97.7	10	3.4	4.2	0.3	46	12.5	15.6	20.8
Kb6	40	249.3	10	4.9	2.3	0.9	120	34.4	13.6	25.2
Tt16	40.7	177	9	8.4	5.3	0.4	111.3	31.8	18.6	21.1
Mean	25.8	120.1	10.5	4.7	13.3	0.5	58.4	21.9	12.2	22.4
LSD (P=0.05)	11.9	91.8	1.0	2.8	4.3	0.6	21.9	8.0	2.74	3.54
SE	7.15	55	0.6	1.7	2.6	0.3	13.1	4.8	1.64	2.12
CV %	24.1	45.8	5.8	38.2	19.3	31.1	22.4	22.0	13.4	9.5

* *in vivo* and *in vitro* sporulation expressed as spore concentration (y $\times 10^3$ spores/ml) per mm^2 of lesion and culture, respectively.

[a] Lesion density refers to number of lesions per cm^2 of leaf.

Table 3. Variation in angular leaf spot severity, AUDPC, incubation period, lesion size and sporulation of Andean, Afro-Andean and Mesoamerican isolates of *Phaeoisariopsis griseola*

Virulence group	ALS Severity (%)	AUDPC	Incubation period (days)	Lesion size (mm^2)	Sporulation *in vivo* ($\times 10^3$)	Sporulation *in vitro* ($\times 10^3$)
Andean	19.5	92.5	13	4.4	39.7	10.0
Afro-Andean	22.5	102.2	11	5.1	50.7	27.4
Mesoamerican	33.5	162.6	8	4.6	78.8	23.7
Mean	25.2	119.1	11	4.7	56.4	20.4
LSD (P=0.05)	7.13	35.1	0.49	1.32	6.9	3.43
SE	3.57	17.6	0.24	0.66	3.45	1.71
CV%	14.2	14.8	2.3	14.0	6.1	8.4

Table 4. Variation in ALS severity, AUDPC, incubation period, lesion development and sporulation of two genetically different groups of *Phaeoisariopsis griseola*

Genetic group	ALS Severity (%)	AUDPC	Incubation period (days)	Lesion size (mm^2)	Sporulation *in vivo* ($\times 10^3$)	Sporulation *in vitro* ($\times 10^3$)
AFLP group I	31.1	133.4	9	5.3	72.7	19.7
AFLP group II	21	99.9	11	4.63	50.2	24.8
Mean	26	116.6	10	4.97	61.4	22.3
LSD (P=0.05)	5.7	48.7	0.61	1.86	13.0	2.93
SE	2.52	21.5	0.27	0.82	5.73	1.29
CV%	9.7	18.4	2.6	16.5	9.3	5.8

The area under disease progress curve (AUDPC) differed significantly (P<0.05) among the isolates (Figure 1). Mesoamerican isolate kb6 had the highest AUDPC value (249.3) whereas Andean isolate eb11 had the lowest value of 31.1 (Table 2). On the average, Mesoamerican isolates had significantly higher AUDPC whereas Andean isolates had the lowest (Table 3). The shape of the disease progress curve also differed among the isolates and reflected the virulence level of the isolates (Figure 2). Less virulent isolates had a lower AUDPC and had a prolonged incubation period. For example, isolate eb11 had a long incubation period of 13 days and also had a low AUDPC value of 31.1 as compared to isolate kb6 which had a shorter incubation period of 10 days and a high AUDPC value of 249.3.

Disease severity was positively correlated to lesion density (r = 0.499, P = 0.05), sporulation *in vivo* (r = 0.411) and sporulation *in vitro* (r = 0.441) (Table 5). Similarly, there was a weak positive correlation between disease severity and lesion size, number of conidiophores per synnema, and synnemata per lesion. There was no significant correlation (r = 0.081, P = 0.05) between disease severity and rate of lesion expansion. Disease severity was negatively correlated (r = -0.434) to incubation period, whereby isolates with a shorter incubation period generally induced more disease. Likewise, there was a negative correlation (r = -0.307) between AUDPC and length of incubation period. Incubation period was negatively correlated to rate of *in vitro* and *in vivo* sporulation (r = -0.145 and -0.461 respectively). The rate of *in vivo* sporulation was positively correlated to rate of *in vitro* sporulation (r = 0.251), number of synnemata per lesion (r = 0.360) and number of conidiophores per synnemata (r = 0.379).

Variation in symptoms induced by *P. griseola* isolates

The symptoms induced by *P. griseola* isolates on leaves of the different bean cultivars varied extensively in terms of size, shape and time of appearance. Some isolates induced disease symptoms as early as six days after inoculation as exemplified by isolates tt17 and mk19, whereas others induced the first symptoms on the 15th day after inoculation as in the case of isolate eb2. The first symptoms developed on the primary leaves

as circular lesions that were initially light grey but later turned dark grey to brown. On the trifoliates, symptoms ranged from small typical angular lesions clearly delimited by veins and veinlets to large coalescing lesions.

As the disease progressed, lesions enlarged very fast and attained larger sizes. In certain pathogen-cultivar interactions, the lesions were accompanied by a spreading burning reaction that gave the leaves a leathery appearance. The type and size of lesions induced could not be attributed to a particular *P. griseola* isolate or bean cultivar. For example, isolate eb1 induced large coalescing lesions in some of the cultivars whereas in others, the lesions were small and did not coalesce. Isolate eb26, on the other hand, induced small typical clearly delimited ALS lesions in all the cultivars except Mwitemania GLP-X.92 and G2858 where the lesions were not well defined. This isolate caused heavy defoliation commencing from the 14th day after inoculation.

Some host-pathogen interactions resulted in non-typical angular leaf spot symptoms as exemplified by the almost circular lesions induced in trifoliates of cultivar G2858 by isolate mk6. The same isolate induced clearly delimited angular lesions that tended to only develop along the veins and veinlets in cultivar Flor de Mayo. Some *P. griseola* isolates induced lesions that were surrounded by a chlorotic halo whereas others caused extensive chlorosis even though the percentage necrotic leaf area was small.

Table 5. Correlation of angular leaf spot severity, disease progress, incubation period and sporulation rate of *Phaeoisariopsis griseola* isolates

	Disease severity	AUDPC	Incubation period	Lesion size	Lesion density	Rate of lesion expansion	Sporulation *in vivo*	Sporulation *in vitro*
AUDPC	0.840							
Incubation period	-0.434	-0.307						
Lesion size	0.226	0.366	0.184					
Lesion density	0.499	0.409	-0.666	-0.352				
Rate of lesion expansion	0.081	0.159	0.107	0.386	-0.252			
Sporulation *in vivo*	0.411	0.468	-0.461	0.332	0.178	0.251		
Sporulation *in vitro*	0.441	0.460	-0.145	0.027	0.224	0.200	0.251	
Synnemata/ lesion	0.249	0.291	-0.222	0.105	0.301	0.069	0.360	0.027
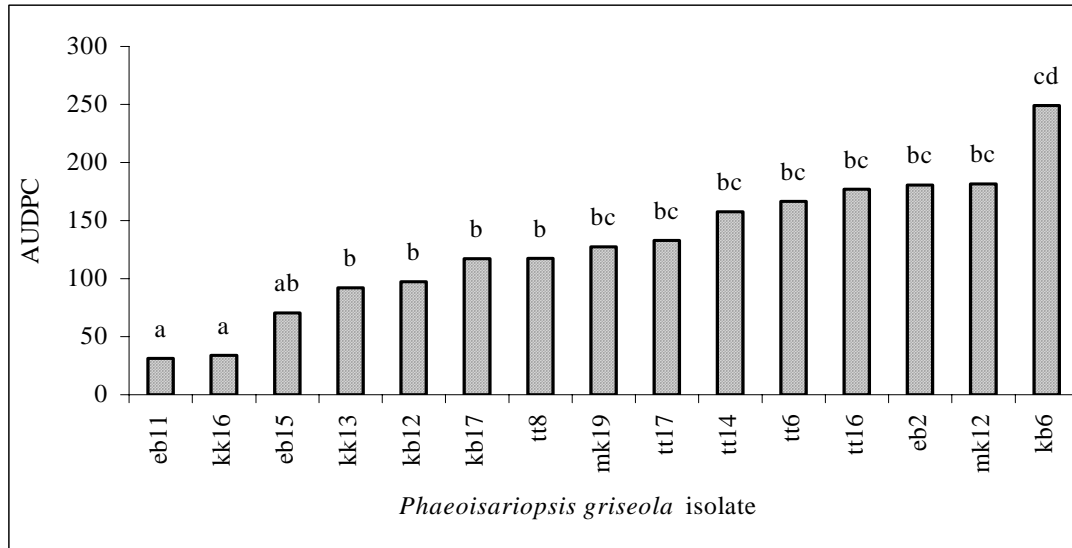
Figure 1. Area under disease progress curve (AUDPC) of 15 isolates of *Phaeoisariopsis griseola* with varying levels of virulence
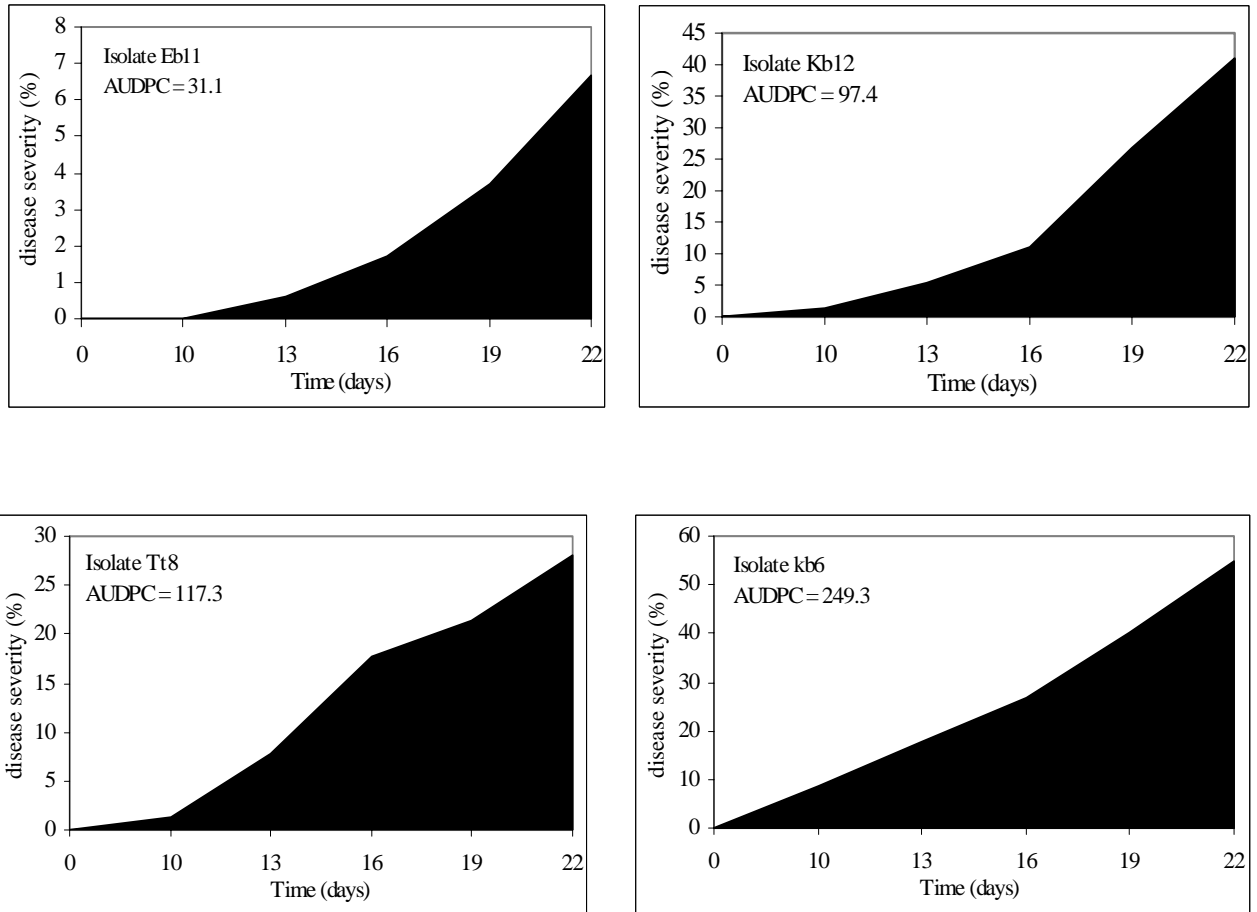
Figure 2. Variations in the shape of disease progress curve (AUDPC) of representative *Phaeoisariopsis griseola* isolates with varying levels of virulence

In all the compatible interactions, dark synnemata were visible on the underside of the leaves starting as early as 10 days after inoculation. Symptoms on petioles, stems and pods did not show notable variations. On petioles and stems, lesions were dark brown and elongated and developed much later than on leaves. Lesions that developed on pods were large, roughly circular and reddish brown. The underlying seeds were

shrivelled and discoloured, often showing brown patches.

DISCUSSION
Variation in aggressiveness of *P. griseola* isolates

The disease reactions induced by isolates of *P. griseola* on susceptible bean cultivars varied significantly in terms of severity, length of incubation period, area under disease progress curve (AUDPC), lesion development and rate of sporulation. These parameters also varied significantly between virulence and genetically defined groups of *P. griseola*. Disease severity was negatively correlated (r = -0.434) to length of incubation period, whereby isolates with a shorter incubation period generally induced more disease. These results are in agreement with those of Buruchara (1983) who observed an association between high angular leaf spot severity levels and shorter incubation period. Disease severity and incubation period are important measures of aggressiveness. According to Van der Plank (1968), races of a pathogen that differ in severity of their pathological effects but do not interact differentially with the host varieties are said to differ in aggressiveness. On a susceptible host (and frequently also in artificial culture) some isolates of a pathogen may grow and/or reproduce more rapidly than others and such isolates are said to be highly aggressive (Manners, 1982). Mesoamerican isolates had a shorter incubation period and higher disease severity levels and are, therefore, more aggressive than Andean and Afro-Andean isolates.

The results of this study indicate that, based on disease severity and length of incubation period, isolates of *P. griseola* can be grouped into three different categories. The highly aggressive group comprises of isolates inducing disease scores of over 25% within a shorter incubation period of less than seven days. The less aggressive group includes isolates that induce very low disease levels and have a prolonged incubation period of more than twelve days. Isolates with an incubation period of more than seven days but shorter than 12 days and inducing over 10% disease severity would be considered as moderately aggressive.

Mesoamerican isolates had significantly higher AUDPC than Andean and Afro-Andean isolates and this further supports the conclusion that they are more aggressive. When *P. griseola* isolates were grouped into genetically different groups based on their AFLP fingerprints, AFLP group I (corresponding to Mesoamerican isolates) was found to be more aggressive than AFLP group II (corresponding to Andean and Afro-Andean isolates). This indicates some level of correlation between genetic makeup and aggressiveness of the isolates. Disease severity was positively correlated to rate of sporulation and these two factors are important in development of disease epidemics. Isolates that induce high infection within a shorter period of time and sporulate heavily would produce a lot of secondary inoculum within the same cropping season resulting in severe crop damage. The polycyclic pathogens that usually cause rusts, mildews and leaf spots are responsible for most of the sudden and catastrophic plant-disease epidemics in the world (Agrios, 1997). Premature defoliation that mainly commences at flowering and beginning of pod filling stage is reported to be the main cause of crop losses attributed to angular leaf spot (Mwang'ombe *et al.*, 1994). Due to reduced foliage, the little food manufactured by the plant is directed towards the plant up-keep rather than pod development and filling.

P. griseola isolates induced lesions of varying types and sizes but there was no significant difference in these parameters between different virulence and genetic groups of the pathogen. Disease severity was more positively correlated to lesion density (r = 0.499) than to lesion size (r = 0.226) and rate of expansion (r = 0.081). This implies that the virulence level of an isolate depends to a greater extent on the number of lesions per unit area rather than the size of lesions and their rate of expansion. The weak positive correlation between lesion size and disease severity noted in this study is supported by reports of Correa-Victoria (1988). He observed no significant correlation between disease severity and lesion size and a low negative correlation between lesion size and average number of lesions. He concluded that although the average lesion size varied greatly, this parameter was independent of pathogenicity but was highly dependent on the host cultivar. Liebenberg and Pretorius (1997) indicated that lesion size may be related to the origin of the host cultivar, the isolate involved and the interaction between the two.

Lesion size is a useful parameter for grouping isolates of various pathogens into pathotypes. It has been used with success on, among others, many rust fungi, especially bean rust (Harter and Zaumeyer, 1941, Davinson and Vaughan, 1963), the small grain rusts (Roelfs *et al.*, 1992) and sunflower rust (Rashid, 1991). Several attempts have previously been made to associate lesion size with pathogenic differences in *P. griseola* (Liebenberg and Pretorius, 1997) but no significant correlations have been reported.

Verma and Sharma (1984) observed two types of ALS lesions but found no significant differences in their number and size. In the present study, significant differences were observed in the density and size of lesions induced by some of the isolates but there was no significant difference in these parameters among the different virulence and genetic groups of *P. griseola*. This implies that grouping isolates based on lesion size would not correspond to virulence and/or genetic groups of the pathogen. It is, therefore, not possible to reliably use lesion size to characterise isolates of the angular leaf spot pathogen.

There was a significant difference in the rate of *in vivo* and *in vitro* sporulation among the isolates and also between the different pathogen groups. As expected, isolates sporulated more heavily on host than in culture. There was an almost equal positive correlation between *in vivo* sporulation and number of conidiophores per synnema and synnemata density (r = 0.379 and r = 0.360, respectively), implying that these two parameters are equally important in determining the rate of sporulation. The rates of *in vivo* and *in vitro* sporulation were positively correlated to disease severity and AUDPC. Therefore, rate of sporulation can be used as a measure of aggressiveness in *P. griseola*. *In vitro* sporulation is easy to determine in the laboratory and can be used to predict how aggressive an isolate would be on the host. This would assist in grouping isolates into different virulence categories, especially where quick decisions need to be made on isolate selection for germplasm screening. Mesoamerican isolates sporulated more heavily than Andean and Afro-Andean isolates and this observation further support the conclusion that Mesoamerican isolates are more aggressive.

A lot of discrepancies exist in determining aggressiveness of a pathogen. Correa-Victoria (1988) measured aggressiveness of *P. griseola* as the number of days after inoculation until 50% of lesions contained spores. He also measured sporulation capacity, expressed as the number of spores produced by mm^2 of lesion. In the present study, disease severity, AUDPC, length of incubation period, and the rate of *in vivo* and *in vitro* sporulation were correlated and varied significantly among isolates as well as between virulence and genetically defined groups of *P. griseola*. All these parameters are important factors in angular leaf spot development and should, therefore, be considered when measuring aggressiveness of the pathogen. The variation in aggressiveness between groups of *P. griseola* isolates regardless of whether the grouping was based on virulence or genetic differences implies that this parameter can be used to characterise isolates of the pathogen. However, further evaluations should be done on a large number of *P. griseola* isolates to confirm the usefulness of aggressiveness as a pathogen characterisation tool. One of the setbacks to this approach, however, is that although aggressiveness may provide valuable information on pathotype differences, it can only be used where evaluations are done in controlled uniform environments, a condition that is usually difficult to achieve in most glasshouses.

Variation in symptoms induced by *P. griseola* isolates

Symptoms induced by isolates of *P. griseola* on leaves in different bean cultivars varied extensively in size, shape and time of appearance. Most of the interactions produced typical angular leaf spot symptoms on leaves, petioles, pods and stems. Some interactions, however, resulted in atypical almost circular lesions on trifoliate leaves. Similar observations had been made by Hocking (1967) who reported the occurrence of a particularly virulent *P. griseola* isolate in Tanzania that caused circular lesions on the trifoliate leaves. Deighton (1990) also reported the existence of three specimens in the International Mycological Institute (IMI) culture collection that caused circular lesions. In the present study, certain host-pathogen interactions resulted in limited chlorosis whereas others were accompanied by extensive chlorosis even though the percent disease severity was low. Extensive chlorosis, just like necrosis, results in substantial reduction of photosynthetic capacity of the plant (Stenglein *et al.*, 2003), leading to low yields.

The wide variation in leaf symptoms induced by different isolates of *P. griseola* reported in this study could not be attributed to any particular host cultivar or pathogen isolate. The type of symptom development was also not correlated to aggressiveness of the isolates. Thus, the type and extent of symptom development is probably a function of the host-pathogen interaction. No significant variations were noted in symptoms induced on petioles, stems and pods. Therefore, although a lot of variations occur in symptoms induced by isolates of *P. griseola*, symptomatology cannot be used to characterise isolates of the pathogen.

References

Agrios, G.N. 1997. Plant Pathology, 4th edition. Academic Press, New York. Pp 61.

Andrivon, D. 1993. Nomenclature for pathogenicity and virulence: The need for precision. *Phytopathology* 83: 889–890.

Anonymous. 1978. Bean Program Annual Report for 1977. CIAT, Cali, Colombia. Pp1–85.

Barros, O.R., Cardona, O.C., Cardenosa, R. and Skiles, R.L. 1958. The control of angular leaf spot in Colombia. FAO Plant Protection Bulletin 6: 97–101.

Boshoff, W.P.H., Swart, W.J., Pretorius, Z.A., Liebenberg, M.M. and Crous, P.W. 1996. Isozyme variability among isolates of *Phaeoisariopsis griseola* in southern Africa. *Plant Pathology* 45: 344–349.

Bruzzese, E. and Hasan, S. 1983. A whole leaf clearing and staining technique for host specificity studies of rust fungi. *Plant Pathology* 32: 335–338.

Buruchara, R. A. 1983. Determination of pathogenic variation in *Isariopsis griseola* Sacc. and *Pseudomonas syringae pv. phaseolicola* (Burk, 1926) Young, Dye and Walkie 1978. Ph.D. Thesis, University of Nairobi, Faculty of Agriculture, 188pp.

Busogoro, J.P., Jijakli, M.H. and Lepoivre, P. 1999. Virulence variation and RAPD polymorphism in African isolates of *Phaeoisariopsis griseola* (Sacc.) Ferr., the causal agent of angular leaf spot of common bean. *European Journal of Plant Pathology* 105: 559–569.

Chacon, M.I., Jara, C., Castellanos, G., Posso, C.E., Buruchara, R.A., Cuasquer, J.B. and Pastor-Corrales, M.A. 1997. Genetic diversity and relation between common bean angular leaf spot fungus isolates from Africa and South America: Genetic improvement implications. Annual Report. Bean Improvement Cooperative. 40: 122–123.

CIAT. 1996. Bean Program Annual Report for 1994; Working Document No. 162. Centro Internacional de Agricultura Tropical. pp 38.

CIAT. 1997. Bean Program. Pages 123–124 in: Annual Report. Cali, Colombia.

Correa-Victoria, F. J. 1988. Pathogenic variation, production of toxic metabolites and isozyme analysis of *Phaeoisariopsis griseola* (Sacc.) Ferr. PhD Dissertation, Michigan State University, East Lansing, M.I. USA. 154 pp.

Correa, F.J. and Saettler, A.W. 1987. Angular leaf spot of red kidney beans in Michigan. *Plant Disease* 71: 915–918.

Davison, A.D. and Vaughan, E.K. 1963. A simplified method for identification of races of *Uromyces phaseoli* var. *phaseoli*. *Phytopathology* 53: 456–459.

Deighton, F.C. 1990. Observations on *Phaeoisariopsis.* Mycological Research 94:1096–1102.

Gomez, K.A. and Gomez, A.A. 1984. Statistical Procedures for Agricultural Research, 2nd Edition, John Wiley & Sons, Singapore, the Philippines. Pp 680.

Guzman, P., Gilbertson, R.L, Nodari, R., Johnson, W. C, Temple, S. R, Mandala, D., Mkandawire, A. B.C. and Gepts, P. 1995. Characterisation of variability in the fungus *Phaeoisariopsis griseola* suggests coevolution with the common bean (*Phaseolus vulgaris*). *Phytopathology* 85: 600–607.

Guzman, P., Gepts, P., Temple, S., Mkandawire, A.B.C. and Gilbertson, R.L. 1999. Detection and differentiation of *Phaeoisariopsis griseola* isolates with the polymerase chain reaction and group-specific primers. *Plant Disease* 83:37–42.

Harter, L.L. and Zaumeyer, W.J. 1941. Differentiation of physiological races of *Uromyces phaseoli typica* on bean. *Journal of Agricultural Research* 62: 717–731.

Hocking, D. 1967. A new virulent form of *Phaeoisariopsis griseola* causing circular leafspot of french bean. *Plant Disease Reporter* 51: 276–278.

Liebenberg, M.M. and Pretorius, Z.A. 1997. A review of angular leaf spot of common bean (*Phaseolus vulgaris* L.). *African Plant Protection* 3: 81–106.

Mahuku, G.S., Henriquez, M.A., Munoz, J. and Buruchara, R. A. 2002a. Molecular markers dispute the existence of the Afro-Andean group of the bean angular leaf spot pathogen, *Phaeoisariopsis griseola*. *Phytopathology* 92: 580–589.

Mahuku, G.S., Jara, C., Cuasquer, J.B. and Castellanos, G. 2002b. Genetic variability within *Phaeoisariopsis griseola* from Central America and its implication for resistance breeding of common bean. *Plant Pathology* 51: 594–604.

Manners, J.G. 1982. Principles of Plant Pathology. Cambridge University Press, Cambridge. Pg 5.

Mwang'ombe, A. W., Kimani, P. M. and Kimenju, J.W. 1994. Evaluation of advanced bean lines for resistance to 6 major diseases in Kenya. A paper presented during the workshop for Bean Research Collaborators. Thika, March 1994.

Pastor-Corrales, M.A., Jara, C. and Singh, S.P. 1998. Pathogenic variation in, sources of, and breeding for resistance to *Phaeoisariopsis griseola* causing angular leaf spot in common bean. *Euphytica* 103: 161–171.

Rashid, K.Y. 1991. Incidence and virulence of *Puccinia helianthi* on sunflower in western Canada during 1988-1990. *Canadian Journal of Plant Pathology* 13: 356–360.

Roelfs, A.P., Singh, R.P. and Saari, E.E. 1992. Rust diseases of wheat: Concepts and methods of disease management. CIMMYT, Mexico.

Saettler, A.W. 1991. Angular leaf spot. In: Compendium of Bean Diseases. Hall R. (Ed.). APS press, St. Paul, M.N. Pp 15–16.

Shaner, G. and Finney, R.E. 1977. The effect of nitrogen fertilisation on the expression of slow-mildewing resistance in knox wheat. *Phytopathology* 67: 1051–1056.

Stenglein, S., Ploper, L.D., Vizgarra, O. and Balatti, P. 2003. Angular leaf spot: A disease caused by the fungus *Phaeoisariopsis griseola* (Sacc.) Ferraris on *Phaseolus vulgaris* L. *Advances in applied microbiology* 52: 209–243.

Van der Plank, J.E. 1968. Disease resistance in plants. Academic Press, New York.

Verma, B. R. and Sharma, S.L. 1984. Variability in *Phaeoisariopsis griseola* the causal agent of angular leaf spot of beans. *Indian Phytopathology* 37: 580–581.

Wagara, I.N. 1996. Pathogenic variability in *Phaeosariopsis griseola* (Sacc.) Ferr. and resistance of *Phaseolus vulgaris* L. to angular leaf spot. M.Sc. Thesis, University of Nairobi. 108 pp.

Wagara, I.N., Mwang'ombe, A.W. and Siboe, G.M. 1999. Colonisation of resistant and susceptible bean tissues by *Phaeoisariopsis griseola* (Sacc.). Ferr. *African Crop Science Journal* 7 (4): 523–530.

Wagara, I.N., Mwang'ombe, A.W., Kimenju, J.W., Buruchara, R.A., Jamnadass, R. and Majiwa, P.A.O. 2004. Genetic diversity of *Phaeoisariopsis griseola* in Kenya as revealed by AFLP and group-specific primers. *Journal of Phytopathology* 152:1–8.

Wortmann, C.S., Kirkby, R.A., Elude, C.A and Allen, D.J. 1998. Atlas of common bean (*Phaseolus vulgaris* L.) production in Africa. CIAT publication No.297, pp 63

Klebsiella oxytoca '10mkr7' stimulates *Striga* suicidal germination in *Zea mays*

Babalola[1,2]* O.O. and G.D. Odhiambo[2]

[1]Department of Microbiology, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria
[2]Department of Botany and Horticulture, Maseno University, P.O. Box 333, Maseno, Kenya
* Corresponding author mailing address: Olubukola O. Babalola, P.O. Box 9536, UI HQ, Ibadan. Tel.:
+2348037035965, E-mail: olubukola_babalola@yahoo.com

ABSTRACT

The *Striga* species are obligate root parasitic plants. Plant growth promoting rhizobacteria could enhance *Striga* suicidal germination. In screen-house studies we quantified the effect of bacterial inoculation with increasing maize plant density on the parasitic weed *Striga hermonthica*. The design used was a randomized complete block with a factorial arrangement (4 plant density levels, 2 *S. hermonthica* infestation levels, artificial and natural, and 2 isolate levels, with and without isolate). Interactions between bacteria and *Striga* count varied significantly (p=0.05). Seed germination differed significantly among plant densities from 6 to 8 WAP in the absence of bacteria. In bacterial inoculated pots significant differences were observed among plant densities from 5 WAP. Maize planted on soil inoculated with *Klebsiella oxytoca* '10mkr7' had higher *Striga* counts at harvest for all plant densities. Significant variation was observed for interactions between bacteria and Striga. These findings suggest that *K. oxytoca* '10mkr7' is a plant growth promoter and could stimulate *Striga* suicidal germination. Hence, there are good prospects for biological control of *Striga* using indigenous rhizosphere *K. oxytoca* '10mkr7'

Keywords: Maize; plant density; *Klebsiella oxytoca*; *Striga hermonthica*; suicidal germination

INTRODUCTION

Studies conducted on plant competition to determine the optimum plant population density of maize (Denazareno *et al.* 1993; Tollenaar *et al.* 1994; Watiki *et al.* 1993) showed that there was no single recommendation for all environments because optimum plant density varies, depending on the environmental factors, such as soil fertility, planting date, and planting pattern (Modarres *et al.* 1998). Proximity to adjacent plants affects maize plant yield (Duncan 1984). Plant population above a critical density has a negative effect on yield per plant due to the effects of interplant competition for light, water, nutrition, and other potential yield-limiting environmental factors (Duncan 1984; Tollenaar *et al.* 1994). Moreover, higher plant densities are encouraged for germplasm improvement in order to facilitate foraging of the unwanted plants (Carena & Cross 2003). *Striga* and *Alectra,* which are agriculturally important genera of the family *Scrophulariaceae* originated from Africa (Vasudeva & Musselman 1987). The purpose of this work was to test the hypothesis that an increased host root system will support higher numbers of *Striga hermonthica* in the presence of potential stimulatory rhizosphere bacteria, with a view to developing effective biological control programs.

MATERIALS AND METHODS

Seeds of the local maize landrace 'Nyamula' from western Kenya were stored in plastic containers in the refrigerator at 7^0C. Seeds of *Striga hermonthica*, collected at Kenya Agricultural Research Institute (KARI)-Kisumu), maize fields (cultivars unknown) during the 2000 long rainy season, were surface sterilized in 1% NaOCl solution for 5 min with continuous stirring. Several changes of sterile water were used to remove the sterilant from the seeds and discarded the floating seeds. Air-dried seeds were stored in small bottles for use when required.

The bacterium '10mkr7' was isolated from the rhizosphere of sorghum plant (local variety Mokwa) in 1998. It had been maintained on 35% glycerol at -80^0C and cloned in Petri dishes using King's Medium B (15g agar, 15g Proteose peptone, 1.5g K_2HPO_4, 1.5g $MgSO_4$, 12.6g glycerol, 1L of distilled water). Incubation was at 28 ± 1^0C for 24 hours. Experiments were planted on 31st January, and 16th March, 2001 at KARI-Kisumu where the soils are naturally infested with *Striga*. *Klebsiella oxytoca* suspended in distilled water was used to inoculate planting holes, at approximately 2 cm deep and 1 cm wide, at a rate of 7.35 x 10^7 cfu ml^{-1} pot^{-1} at planting. Pots that served as blank control received sterile distilled water only.

Experimental design was a randomized complete block with a factorial arrangement (4 plant density levels, 1, 2, 4 and 6; 2 *Striga* infestation levels, artificial and natural; and 2 isolate levels, with and without isolate) resulting in 4 × 2 × 2 = 16 treatment

combinations. There were 32 pots in the first experiment and 64 pots in the second. Each pot (32 × 32 cm) was sown at 4 density levels (1, 2, 3, or 4 maize plants pot^{-1}). Pots were arranged on screen-house floor. The experiment was performed twice. There were only two replications in the first experiment because of limited resources but four replications were performed in the second experiment The soil used was naturally infested but was also artificially infested (50 mg pot^{-1}) so that relative performance could be assessed. Precaution was taken in the management of the two infestation levels to avoid cross contamination by planting the naturally infested soil first before the planting of artificially infested soil. A period of 14 days was allowed for *S. hermonthica* preconditioning. At 2 weeks after planting (WAP), the seedlings were thinned to 1, 2, 4 or 6 plants pot^{-1}, depending on the allocated root density.

Data were collected on number of days to *S. hermonthica* emergence. At the end of the growing period, visual rating of *S. hermonthica* growth was evaluated on a scale of 1 to 6 at 12 WAP. Where, 1 = small plants, no flowering; 2 = medium plants, some flowering; 3 = medium plants, full flowering 4 = large plants, full flowering; 5 = large plants, some capsules; 6 = large plants, full capsules. At 9 WAP, maize damage scores were evaluated on a scale of 1 to 9 (1 = no symptoms and 9 = all plants dead or dying). Details of the rating are as follows (Kim 1994), 1 = Normal plant growth, no visible symptoms, 2 = Small and vague. Purplish-brown leaf blotches visible, 3 = Mild leaf blotching, with some purplish-brown necrotic spots, 4 = Extensive blotching and mild wilting. Slight but noticeable stunting and reduction in ear and tassel size, 5 = Extensive leaf blotching, wilting, and some scorching. Moderate stunting, ear and tassel size reduction, 6 = Extensive leaf scorching with mostly grey

necrotic spots. Some stunting and reduction in stem diameter, ear size, and tassel size, 7 = Definite leaf scorching, with grey necrotic spots, and leaf wilting and rolling. Severe stunting and reduction in stem diameter, ear size, and tassel size, often causing stalk lodging, brittleness, and husk opening at a late-growing stage, 8 = Definite leaf scorching, with extensive grey necrotic spots. Conspicuous stunting, leaf wilting, rolling, severe stalk lodging, and brittleness. Reduction in stem diameter, ear size, and tassel size, 9 = Complete scorching of all leaves, causing premature death or collapse of host plant and no ear formation. The maize plants from each pot were removed and dried at 70°C to a constant weight. *Striga* count data were subjected to square root transformation for analysis. Correlations were calculated between the variables measured. Combined analysis of variance was conducted on data between experimental dates to identify a high and stable average effect. The linear model of SAS version 9.1 (SAS 2003) was used to analyze the data collected. Means were separated using a Tukey HSD test. Significance level was P = 0.05 in all tests except otherwise stated.

RESULTS

Mean squares of *Striga* count from 5 to 8 WAP were highly significant (P<0.01) (Table 1). At 5 WAP no interaction was significant (Table 1). At 6, 7 and 8 WAP the mean squares for *Striga* counts for Bacteria × *Striga*, Plant density, and *Striga* × plant density were significant. As bacteria became significant, the interaction of bacteria with *Striga* also became significant (Table 1). All through the experiment, *Striga* emergence count increase from 4 to 8 WAP (Table 2). A similar trend of *Striga* counts was observed between host plant population densities: with *Striga* counts increasing as plant population increased (Table 2).

Table 1: Mean squares and significant levels from analyses of variance of *Striga* emergence count at two bacteria levels, two *Striga* levels and four plant populations

Source	Df	Mean squares and significance levels (WAP)			
		5	6	7	8
Corrected total	95				
Bacteria	1	0.38	3.32	17.61**	28.71**
Striga	1	46.70**	202.45**	402.71**	497.98**
Bacteria x *Striga*	1	0.69	3.30*	14.04**	19.46**
Plant density	3	4.00	6.48**	4.92*	18.62**
Striga x plant density	3	3.89	6.32*	4.42*	11.83*
Error	80	0.54	1.01	1.08	1.24
R-square		0.64	0.75	0.84	0.85
CV (%)		51.4	44.9	32.41	29.56

Table 2: Mean *Striga* emergence counts of maize at two bacteria levels, two *Striga* levels and four plant populations from week 5 to 9 after planting

Treatments	Levels	*Striga* emergence counts (WAP)[a]				
		5	6	7	8	9
Bacteria	Control	1.36a	2.14a	2.78b	3.22b	3.40b
	Inoculated	1.49a	2.51a	3.63a	4.31a	4.25a
	LSD (P=0.05)	0.30	0.42	0.42	0.45	0.43
Striga	Natural	0.73b	0.87b	1.16b	1.49b	1.89b
	Artificial	2.12a	3.78a	5.25a	6.04a	5.75a
	LSD (P=0.05)	0.29	0.42	0.42	0.45	0.43
Density (Plants/pot)	1	0.71b	1.72c	2.64b	3.19c	3.33b
	2	1.17b	2.08bc	3.04ab	3.51bc	3.81ab
	4	1.68a	2.64ab	3.55a	4.30a	4.34a
	6	1.85a	2.86a	3.59a	4.06ab	3.81ab
	LSD (P=0.05)	0.42	0.60	0.59	0.64	0.61

[a] Values presented are log-transformed data.

For plant population of 6, *Striga* emergence count differed significantly among plant densities from 6 to 8 WAP in the absence of *Klebsiella oxytoca* (Table 3). In inoculated pots, *Striga* counts at 7 WAP were not significantly different among plant densities under infestation. Data of *Striga* emergence only give a partial view of the complex interactions of factors (Kim 1994). Plants from soil inoculated with *K. oxytoca* had higher *Striga* counts at harvest for all plant densities examined (Table 3). In the non-inoculated control all plant densities examined showed a reduction in the number of *Striga* shoots (Table 3).

Table 3: Mean squares and LSD for *Striga* emergence counts made with or without artificial bacterial inoculation in the soil at planting for four plant populations

Bacterial treatment	Plant population	*Striga* emergence count (WAP)[a]			
		5	6	7	8
Inoculated	1	1.03b	2.60b	5.18a	6.24bc
	2	1.57b	3.25ab	5.13a	5.86c
	4	3.16a	5.33a	6.83a	8.18a
	6	3.32a	5.41a	7.11a	7.88ab
	LSD(0.05)	1.34	2.17	2.03	1.90
Non-inoculated control	1	1.55a	2.77b	3.69b	4.24b
	2	1.60a	3.06b	4.12b	4.83ab
	4	2.16a	3.46ab	4.55ab	5.38ab
	6	2.59a	4.34a	5.40a	5.72a
	LSD(0.05)	1.14	1.15	1.18	1.35

[a] Means with the same letter within column of the same bacterial treatment level are not significantly different from each other (P = 0.05) according to Tukey HSD test.

K. oxytoca '10mkr7' significantly (P < 0.05) enhanced *Striga* fresh weight. For the number of days to *Striga* emergence, only *Striga* infestation was significant (P < 0.001) among the main effects. Significant variation was observed for interactions of bacteria X *Striga* infestation for *Striga* fresh weight (P < 0.01) and maize damage score (P < 0.01), (Table 4). The interactions of bacteria and plant density were not significant and therefore have not been presented.

Data from the repeated experiments showed that the presence of bacteria increased the host plant yield components over the non-inoculated control with consequent significant reduction in maize damage score (Table 5).

Table 4: Mean squares from ANOVA of *Striga* indices and maize damage score from maize at four plant densities under screen-house conditions

Source	Df	Mean squares			
		Days to emergence	Visual rating of *Striga* (1 to 6)[b]	*Striga* fresh weight	Maize damage score (1 to 9)[c] 9 WAP
Corrected total	95				
Bacteria	1	2.99	1.50	2524.06*	2.04
Striga	1	8361.01***	42.67***	21454.75***	330.04***
Bacteria x *Striga*	1	2.16	0.37	3627.89**	7.04**
Plant density	3	96.91	2.74**	385.35	3.26*
Striga x plant density	3	22.92	1.19	596.18	1.04

Df = degree of freedom
[a]*, **, *** Significant at p = 0.05, 0.01 and 0.001, respectively according to Tukey's studentized range (HSD Test).
[b]Visual rating of *Striga* growth (1 to 6): 1 = small plants, no flowering; 6 = large plants, full capsules
[c]Maize damage score on pot basis: 1 = no damage, 9 = highest damage death^{-1}.

Table 5: Maize damage scores and yield components of maize (var. Nyamula) averaged over bacterial treatments, *Striga* infestation treatments and plant densities

Treatments	Levels of treatment	Plant height (cm)	Ear number (no/pot)	Maize damage score (1 to 9)[a]	Ear weight (g)	Stover dry weight (g)
Bacteria	Control	94.79b	0.75a	6.63a	10.06a	48.94b
	Inoculated	113.85a	0.77b	6.33b	10.65a	71.19a
	LSD $_{(0.05)}$	13.13	0.01	0.17	5.03	17.49
Striga infestation	Natural	153.79a	1.33a	4.63b	19.90a	96.30a
	Artificial	54.85b	0.19b	8.33a	0.81b	23.29b
	LSD $_{(0.05)}$	13.13	0.32	0.47	5.03	18.15
Density (Plants/pot)	1	121.04a	0.46a	6.08a	12.85a	51.75a
	2	108.75ab	0.83a	6.25a	13.29a	60.60a
	4	102.20ab	0.83a	6.75a	10.16a	52.87a
	6	85.29b	0.92a	6.83a	5.12a	75.53a
	LSD $_{(0.05)}$	24.49	0.59	0.87	9.38	33.85

[a]Maize damage score on pot basis: 1= no damage, 9 = highest damage
Means with the same letter within column of the same treatment level are not significantly different from each other (P = 0.05) according to Tukey HSD test.

This is particularly true of the artificially infested pots, where the average increases recorded were more than 100% for maize plant height, ear weight, and stover biomass (Table 5).

On the average, a 10.47% decrease was recorded for maize damage score in the inoculated pots. This is to suggest that *K. oxytoca* '10mkr7' confers strength on the host plant by increasing available nutrients (Table 5), as concluded previously by Skipper *et al.* (1996). The increase in yield components brought about by bacteria averaged between 0.05 and 33.9% for the naturally infested pots over the four plant densities (Table 5).

Among the plant densities considered, the effect of plant height was significantly different (Table 5). This is consistent with the report of Singh *et al.* (1991) that plant density is known to affect *Striga* infestation. As plant density increases, plant height decreases (Table 5). The tallest plant height was recorded in the treatment that had one plant per pot, however it supported the lowest ear number. Maize damage score increased as plant density increased from the population of 1/pot to 6/pot though not to a significant extent (Table 5).

Plant density of 6/pot supported the greatest stover biomass but the biomass was not statistically significant. From this study, the optimal plant population density on maize is 2/pot (Table 5). In the presence of bacteria, under artificial *S. hermonthica* infestation, a consistent trend of decreasing yield components with increasing plant population was observed. For example, plant height decreased from 96.00 cm to 58.50 cm as plant population increased from one plant to 6 maize plants/pot. No consistency was observed in the trend of yield components among plant densities in the absence of bacteria (Data not shown).

DISCUSSION

Results from our screenhouse-grown plants are similar to the field findings of Kim (1994) who reported an increase in *Striga* emergence count in maize from 5 to 8 WAP when the highest numbers of emerged *Striga* were observed. The one-week difference in the first day of emergence between our report and that of Kim (1994) could be associated with the *Striga* depth in the soil. The coefficient of variation in *Striga* counts was observed to decrease consistently from 5 to 8 WAP when the host plant was harvested. The most discouraging aspect of our study was the high CV but this CV is associated with *Striga* count data. Kim *et al*. (1998) reported a much higher CV of 61% for *Striga* emergence on their study on Synthetic maize population.

Striga emergence counts varied little between the natural and the artificially infested pots for bacterial effect at 5 to 6 WAP. The insignificant difference observed between the inoculated and the non-inoculated pots for early *Striga* counts (5 WAP) might be due to reduced microbial activity in the subsurface horizons. However, from 5 to 8 WAP, *Striga* counts between the 2 levels of infection had significant differences. In the presence of bacteria, *Striga* counts were greater (Table 2). This was expected and the result is consistent with others findings (Adetimirin *et al*., 2000). The LSD between the naturally infested and the artificially infested pots widened as the plants approached maturity. The *Striga* plants in artificially infested pots outnumbered those in naturally infested pots. Again, the LSD gradually increased from 5 to 8 WAP. Maize plants grown in pots artificially inoculated with bacteria did not support increasing counts of *Striga* shoot to harvest, however the *Striga* count recorded was still significantly different from the number in the non-inoculated pots despite the numerical decrease in the *Striga*

counts. One reason for the reduced efficacy might be that other soil bacteria outgrew the inoculated isolate. However, all evidence points toward the rhizosphere competence of *K. oxytoca* '10mkr7'.

Though *Striga* emergence count reduced for plant population 6 by harvest, the linear trend of increasing *Striga* counts with plant population was still observed. Results from this study support an earlier report by Carsky *et al*., (2000) which stated that increasing maize plant density did not result in lower emerged *S. hermonthica*. In the two instances (January and March) investigated, the environmental soil was not *Striga*-free hence the naturally infested experimental soil. This has been established by another study by the authors. The repeated artificial infestation of the soil in the research station could also help to explain the high *Striga* count observed in the non-infested pots. These results are in agreement with those of Singh *et al*. (1991). A singular factor might be the specific bacteria in question. Previous findings corroborate this research findings have been published by Babalola *et al*. (2002) and Babalola *et al*. (2007). In our experiment, the bacterium seems to be a plant growth promoter. Several factors could be responsible for the performance of the isolate. Among such factors is competition between the introduced isolate and the already established bacteria in the rhizosphere, each of which is likely to have a different biotic potential. Environmental factors might also have contributed to the result obtained by retarding the carrying capacity of the isolate and thereby interfering with their profound need for survival. Several authors (Carsky *et al.* 1998) have reported reduced harvest index as plant density increased. It is evident from the above results that plant height, ear weight, and stover dry matter decrease as the plant density increases from 1 to 6/pot. Increasing the plant density increases the proportion of plants without an ear. This could be due to plant competition for nutrient and available resources in the potted soil. Previous research works (Esechie 1992) confirmed this. Esechie (1992) and (Cox 1996) also previously reported that increasing maize plant density resulted in smaller maize ears.

for reviewing this manuscript and offering suggestions.

REFERENCES

Adetimirin, V.O., Kim, S.K. & Aken'Ova, M.E. 2000 Expression of mature plant resistance to *Striga hermonthica* in maize. *Euphytica* 115, 149-158.

Babalola, O.O., Sanni, A.I, Odhiambo, G.D. & Baldwyn, T. 2007 Plant growth-promoting rhizobacteria do not pose any deleterious effect on cowpea and detectable amounts of ethylene are produced. *World Journal of Microbiology and Biotechnology* **23**, 747-752.

Babalola, O.O., Osir, E.O. & Sanni, A.I. 2002 Characterization of potential ethylene-producing rhizosphere bacteria of *Striga*-infested maize and sorghum. *African Journal of Biotechnology* **1**, 67-69.

Carena, M.J. & Cross, H.Z. 2003 Plant density and maize germplasm improvement in the northern corn belt. *Maydica* **48**, 105-111.

Carsky, R.J., Nokoe, S., Lagoke, S.T.O. & Kim, S.K. 1998 Maize yield determinants in farmer-managed trials in the Nigerian northern Guinea savanna. *Experimental Agriculture* **34**, 407-422.

Carsky, R.J., Berner, D.K., Oyewole, B.D., Dashiell, K. & Schulz, S. (2000) Reduction of *Striga hermonthica* parasitism on maize using soybean rotation. *International Journal of Pest Management* **46,** 115-120.

Cox, W.J. (1996) Whole-plant physiological and yield responses of maize to plant density. *Agronomy Journal* **88,** 489-496.

Denazareno, N.R.X., Madden, L.V. & Lipps, P.E. 1993 Characterization of gray leaf-spot epidemics of maize. *Journal of Plant Disease Protection* 100: *410-425*, 410-425.

Duncan, W.G. 1984 A theory to explain the relationships between corn population and grain yield. *Crop Science* **24**, 1141-1145.

Esechie, H.A. 1992 Effect of planting density on growth and yield of irrigated maize (*Zea mays*) in the Batinah Coast region of Oman. *Journal of Agricultural Science* **119**, 165-169.

Kim, S.K. 1994 Genetics of maize tolerance of *Striga hermonthica*. *Crop Science* **4**, 900-907.

Kim, S.K., Fajemisin, J.M., The, C., Adepoju, A., Kling, J., Badu-Apraku, B., Versteeg, M. Carsky R & Lagoke, S.T.O.1998 Development of synthetic maize populations for resistance to Striga hermonthica. *Plant Breeding* **117**, 203-209.

Modarres, A.M., Hamilton, R.I., Dijac, M., Dwyer, L.M., Stewart, D.W., Mather, D.E. & Smith, D.L. 1998 Plant population density effects on maize inbred lines grown in short season environments. *Crop Science* **38**, 104-108.

SAS 2003 *SAS/STAT User's guide, Release 9.1. Cary, NC*, Cary, NC.

Singh, L., Ndikawa, R. & Rao, M.R. 1991 Integrated approach to *Striga* management on sorghum in North Cameroon. In: *Proc.5th Int. Symp. of Parasitic Weeds* (ed Parker, C.), pp. 223-231. CIMMYT, Nairobi.

Skipper, D.H., Ogg, G.A. Jr. & Kennedy, A.C. (1996) Root biology of grasses and ecology of rhizobacteria for biological control, *Weed Technology.* **10,** 610-620.

Tollenaar, M., Dibo, A.A., Aguilera, A., Weise, S.F. & Swanton, C.J. 1994 Effect of crop density on weed interference in maize. *Agronomy Journal* **86**, 591-595.

Vasudeva, R.M.J. & Musselman, L.J. 1987 Host specificity and physiological 'Strains'. In: *Striga*, Vol. 1 (ed Musselman, L. J.), pp. 13 - 25. CRC press, Boca Raton, Florida, USA.

Watiki, J.M., Fukai, S., Banda, J.A. & Keating, B.A. 1993 Radiation interception and growth of maize/cowpea intercrop as affected by maize plant-density and cowpea cultivar. *Field Crop Research* **35**, 123-133.

Heavy metal tolerance and antibiotic resistance profiles of gram-negative bacteria isolated from Lake Victoria, Kenya

[1*]Boga HI, [2]Okemo PO, [2$]Mwatha WE, [1]Muthanga J, [3]Tsanuo MK and [4]Ikingura JR
[1]Jomo Kenyatta University of Agriculture and Technology, Botany Department, P.O. Box 62000 (00200) Nairobi, Kenya
[2]Kenyatta University, Botany Department, P.O. Box 43844(00100) Nairobi, Kenya
[3]Jomo Kenyatta University of Agriculture and Technology, Chemistry Department, P.O. Box 62000 (00200) Nairobi, Kenya
[4]University of Dar-es-Salaam, Geology Department, P.O. Box 35052 Dar-es-Salaam, Tanzania
*Corresponding author. hboga@fsc.jkuat.ac. [$]Deceased

ABSTRACT

Pollution and increased levels of heavy metals in the Lake Victoria Wetlands are suspected to be negatively influencing the biota in one of the world most important freshwater resources. In this study, fecal contamination of water in the Lake Victoria wetlands was investigated using the standard coliform test. Gram-negative bacteria were isolated by plating on Mackonkey's and EMB media. Isolates were characterized, and their ability to grow in the presence of heavy metals tested. Isolates were also tested for resistance to commonly used antibiotics. The study was conducted both in the dry and wet seasons. Fecal contamination was detected in all tested water samples, as evidenced by the isolation of *Escherichia coli* both in the wet and dry seasons. Coliform counts of above 1100 MPN/100 ml were detected. Ninety-five and forty-five gram-negative bacterial isolates were obtained in the dry season and wet seasons, respectively. Isolates from the dry season were resistant to nalidixic acid (66%), ampicillin (54%), and tetracycline (28%). They were however largely sensitive to gentamycin (95%) and streptomycin (98%); 41.1% percent of the isolates showed multi-drug resistance. A similar antibiotic resistance pattern was detected in the wet season, and 31.1% of the isolates were multi-drug resistant. Many of the isolates were resistant to Zinc and Copper (final conc. 1.6 g/l each), but largely sensitive to mercury (0.05 g/l; 52%) and lead (2 g/l; 89%). Although, tolerance to heavy metals is usually associated with plasmids, which also encode resistance to antibiotics, a direct correlation between antibiotic resistance and heavy metal tolerance by the isolates obtained in this study cannot be established. Further work needs to be conducted to establish the molecular basis of resistance. Constant monitoring of antibiotic resistance levels in bacteria from Lake Victoria wetlands is essential, considering the prevalence of water borne diseases in the area.

Key words: Lake Victoria, fecal pollution, Gram Negative bacteria, Heavy metal tolerance

INTRODUCTION

Lake Victoria is the second largest lake in the world, and has the world's largest freshwater fisheries (Kayombo and Jorgensen 2006). The lake basin supports 30 million people, and threats facing the lake include a rapidly growing population, pollution, overexploitation and climate change. Domestic and industrial waste, solid waste, sediments from soil erosion, and atmospheric deposition are a major source of nutrients. The biodiversity in the catchment area of Lake Victoria is being devastated because of the increased population and associated increase in human activities such as mining, industries and use of agrochemicals. These human activities around Lake Victoria are likely to impact on the water quality and the quality of wetland resources such as fish derived from the area.

There have been several studies on the environmental aspects of mining activities in the Lake Victoria Gold Fields (LVGF) (Ikingura, 1994; Kahatano and Mnali 1995; Landner, 1995; Ikingura and Mutakyahwa, 1995; Kahatano et al., 1997; Ikingura, 1997; Mnali, 1997), mainly looking at the impact of small-scale mining to the immediate surroundings of local communities and not to the wetland ecosystem. Apart from increasing turbidity and suspended load in river water, the processing of gold-ore along rivers caused siltation and diversion of seasonal rivers and streams. The major chemical impacts from the mining operations were

contamination of soil, rivers and air by mercury (Hg) and enhancement of heavy metal load in riverbed sediments. The *aqua regia* leachable heavy metals lead(Pb), Copper (Cu), Cromium (Cr), Cadmium (Cd) and Zinc(Zn) showed enrichment factors of 1.5–16 in contaminated river sediment relative to uncontaminated sediments. The study of gold sites in the Migori Gold Belt of Kenya by Ogola et al. (2002) revealed that the concentrations of heavy metals, mainly Mercury (Hg), Pb and Arsenic (As), are above acceptable levels. Stream sediments had metal concentrations of 3–11075 mg/kg for Pb, 0.014–1.87 mg/kg for As and 0.28–348 mg/kg for Hg. Mine tailings contained much higher levels of the heavy metals than the stream sediments.

Heavy metals have been shown to enhance multi-drug resistance in microorganisms. Genes for metal resistance in bacteria are often plasmid encoded (De Rore et.al. 1994a and De Rore et.al. 1994b) just as is the case with genes for antibiotic resistance. Plasmids such as pJP4 codes for resistance to mercury ions and herbicides (Newby et al., 2000). Different heavy metals resistance patterns have been reported in various microorganisms (Richards et al., 2002, Butcher et al., 2000, and Xiong and Jayaswal, 1998). The release of heavy metals either from industries or mines constitutes a major environmental problem. Heavy metals like vanadium and nickel, which are constituents of

crude oil, are toxic even at low concentrations (Harnandez et al 1998). It has been shown that bacterial isolates, which were resistant to vanadium showed increased resistance to quinolones (ciprofloxacin and norfloxacin), which are crucial in the management of most severe gram-negative infections. Plasmid mediated resistance is often transferred to indigenous populations of microorganisms occurring frequently in natural environments (Newby et al., 2000) thereby enhancing the spread of antibiotic resistance. This has the potential of rendering useful antibiotics harmless to the bacteria they are expected to kill or inhibit during treatment of common bacterial diseases such as typhoid, cholera and dysentery.

Urban effluent has also been shown to result in an increase in the rates of resistance to antibiotics in the riverine bacterial populations which maybe attributed to the discharge of antibiotics in various amounts in the environments as a result of the increasing and often indiscriminate use of antibiotics in medical, veterinary, and agricultural practices (Al-Ghazali et al., 1988, Al-Jebouri, 1985, Bhattacherjee et al.1988, Pathak et al. 1993) or due to increased discharge of heavy metals. River waters are the main receptacles for these pollutants, since they receive the sewage of urban effluents. As rivers are one of the major sources of water,

directly or indirectly, for human and animal consumption, this pollution may contribute to the maintenance and even the spread of bacterial antibiotic resistance (Goni-Urriza et al. 2000).

Heavy metal loading in the wetlands around the lake can occur naturally or artificially, through pollution. The polluting sources include industries, mining activities, and agricultural inputs (chemical fertilizers, insecticides). Metals and other chemicals that reach the wetlands are taken up by fauna and flora or pass through into the lake. Mining activities could also impact on the quality of the water by releasing heavy metal ions into the lake, which then enter the food chain. This study investigated the extent of fecal pollution around Lake Victoria by gram-negative bacteria resistance to antibiotics and heavy metals.

MATERIALS AND METHODS

Study site

Sampling was carried out at 7 stations including, Kasat River, Hippo Point, Winam Gulf, Yala River, Asembo Bay, Kuja River, Kwach River, Migori River and Masara Gold mine. The positions of the sampling stations are indicated in Figure 1. Sampling sites were selected based on accessibility and potential for contamination from mines.
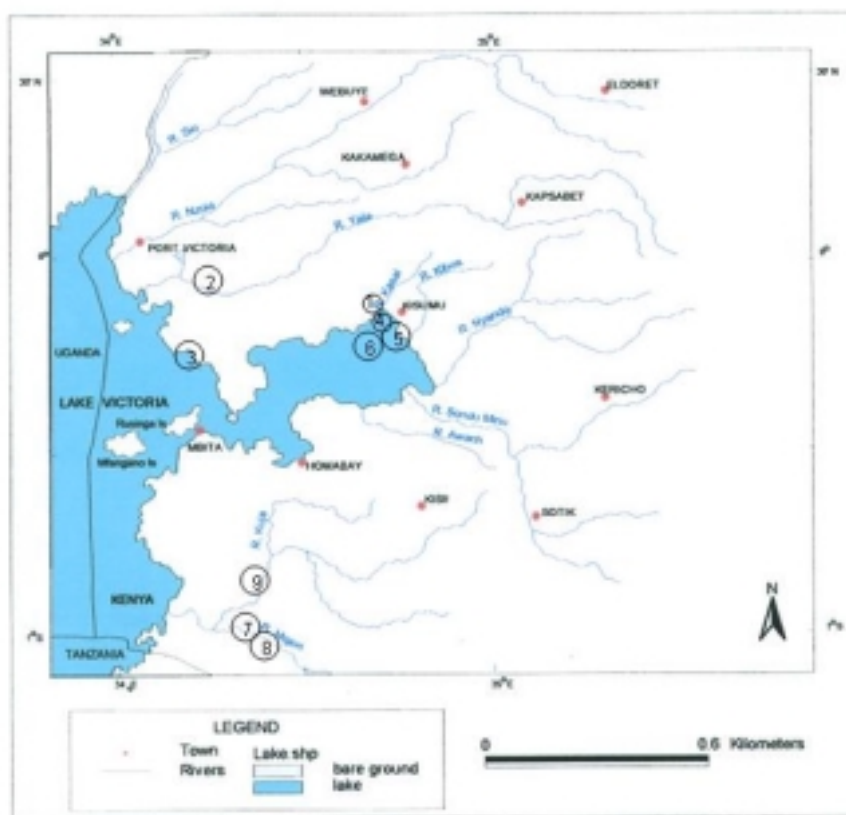

Figure 1. Study site and sampling points along the Kenyan Side of the Lake Victoria Basin

Sampling

Water and sediment samples were collected into sterile plastic bottles, and were stored at –4°C until processing. Sediments were augured at a depth between 0 and 20 cm. For microbiological studies samples were processed within one day of collection. Samples were collected both in the rainy season (May 2004) and dry season (18–22 February 2004)

Culture media

Coliforms were enumerated in MacConkey broth using the standard 3-tube MPN. Enterobacteriaceae in water and soil samples were isolated on Mackonkey Agar (Difco). Total viable cells in water and sediment samples were enumerated on nutrient agar (Difco) plates. Different colony morphotypes were isolated in pure culture and were characterized in terms of colony morphology, cell morphology, gram stain reaction, and biochemical characteristics.

Heavy metal tolerance

To test for tolerance to heavy metals, isolates were grown on nutrient broth medium containing Zn^{2+} (1.6 g/l), Cu^{2+} (1.6 g/l), Cd^{2+} (0.8 g/l), Hg^{2+} (0.05 g/l) or Pb^{2+} (2 g/l). Growth was measured as increase in turbidity with the uninoculated medium containing the respective heavy metal as the control.

Determination of antibiotic tolerance

Isolates were tested for resistance to ampicillin (33 μg), streptomycin (25 μg), gentamycin (10 μg), tetracycline (30 μg), nalidixic acid (30 μg) (High media laboratories, Mumbai India). Inoculum was diluted to correspond to 0.5 MarcFarland standard and then was spread on Mueller Hinton Agar (Difco). Commercial disks impregnated with the above antibiotics were placed on the agar surface and plates were incubated at 37°C for 24 h. Zones of inhibition were recorded and the diameter (mm) measured. Isolates were designated as resistant, sensitive or intermediate as described by National Committee for Clinical Laboratory Standards (2005). *Escherichia coli* (ATCC 25922) was used as a control for potency of antibiotic discs.

RESULTS

Total cell counts and cell diversity

All tested water samples had high densities of viable cells ($> 10^5$ cfu/ml). A total of 95 isolates were obtained. In the dry season, River Kasat had the highest diversity of colony forming units compared to other sampling stations, with 23 morphotypes (Figure 2). In the wet season a total of 45 different colony morphotypes were obtained, with the highest diversity being recorded in Masara goldmine. Bacterial numbers in the wet season were also greater than, 10^5 cells/ml in both water and sediment samples from all sampling stations. The Hippo point deep-site, which was located about 300 m from the shores of the lake had only one morphotype.

Coliform counts

All tested samples except the Hippo Point (deep) showed Coliform counts greater than 1100 cells/ml. The presence of *Escherichia coli* in all tested samples except the Hippo Point (deep) sample was confirmed on EMB plates by the appearance of colonies with a green metallic sheen, and the isolation of Gram-negative rod shaped bacteria, which ferment lactose broth to produce acid and gas. Gram-negative bacteria isolated in the dry season (95 morphotypes) and in the rainy season (45 morphotypes) were tested for tolerance to heavy metals and resistance to antibiotics.

Bacterial tolerance to heavy metals and antibiotics

More than 90% of the isolates obtained in the rainy season were toleterant to Zn^{2+} (1.6 g/l) and more than 60% were tolerant to Cu^{2+} (1.6 g/l) and Cd^{2+} (0.8 g/l) (Figure 3). Majority of the isolates were sensitive to Hg^{2+} and Pb^{2+}. In the dry season almost a similar pattern of tolerance to Zn^{2+}, Cu^{2+} and Cd^{2+} was observed. However, isolates tolerant to Hg decreased, while more isolates were tolerant to Pb^{2+} than was the case during the rainy season.

Bacterial tolerance to Antibiotics

Majority of the isolates (over 90%) in both the rainy and dry seasons were sensitive to streptomycin and gentamycin (Figure 4A and B), while nearly 50% were resistant to ampicillin and nalidixic acid. A large fraction of the isolates, 42.1% in the dry season and 31.1% in the rainy season were resistant to more than 2 antibiotics (Table 2). It was also apparent that 24.3% and 15.4% of the isolates from the dry and rainy seasons, respectively could resist more than 3 drugs.

Table 1. Bacteriological studies of water and sediment samples from various sampling stations on the Kenyan side of Lake Victoria during the dry season (February 2004)

Medium	Sample	Winam	Kisat	Yala	Asembo	Migori	Kuja	Kwach	Masara	Hippo point, deep	Hippo Point shallow
Dry Season											
Coliforms (MPN/100 ml)	W	> 1100	> 1100	> 1100	> 1100	> 1100	> 1100	nd	nd	5	nd
Total Plate Count (cells per ml)	W	> 10^5	> 10^5	nd	> 10^5	> 10^5	> 10^5	> 10^5	> 10^5	< 10^5	> 10^5
	S	> 10^5	> 10^5	nd	> 10^5	nd		> 10^5	> 10^5	nd	> 10^5
MacCkonkey's Agar plates	W	+	+	+	+	-	+	+	+	> 10^3	nd
Wet Season											
Coliforms (MPN/100 ml)	W	> 1100	> 1100	> 1100	> 1100	> 1100	> 1100	>1100	>1100	>1100	>1100
Total Plate Count (cells per ml)	W	> 10^5	> 10^4	> 10^4	> 10^5	> 10^5	> 10^5	> 10^5	> 10^5	> 10^5	> 10^5
	S	> 10^5	> 10^4	> 10^5	> 10^5	> 10^5	> 10^5	> 10^5	> 10^5	nd	> 10^5
Mackonkey's Agar plates	W	+	+	+	+	-	+	+	+	+	+

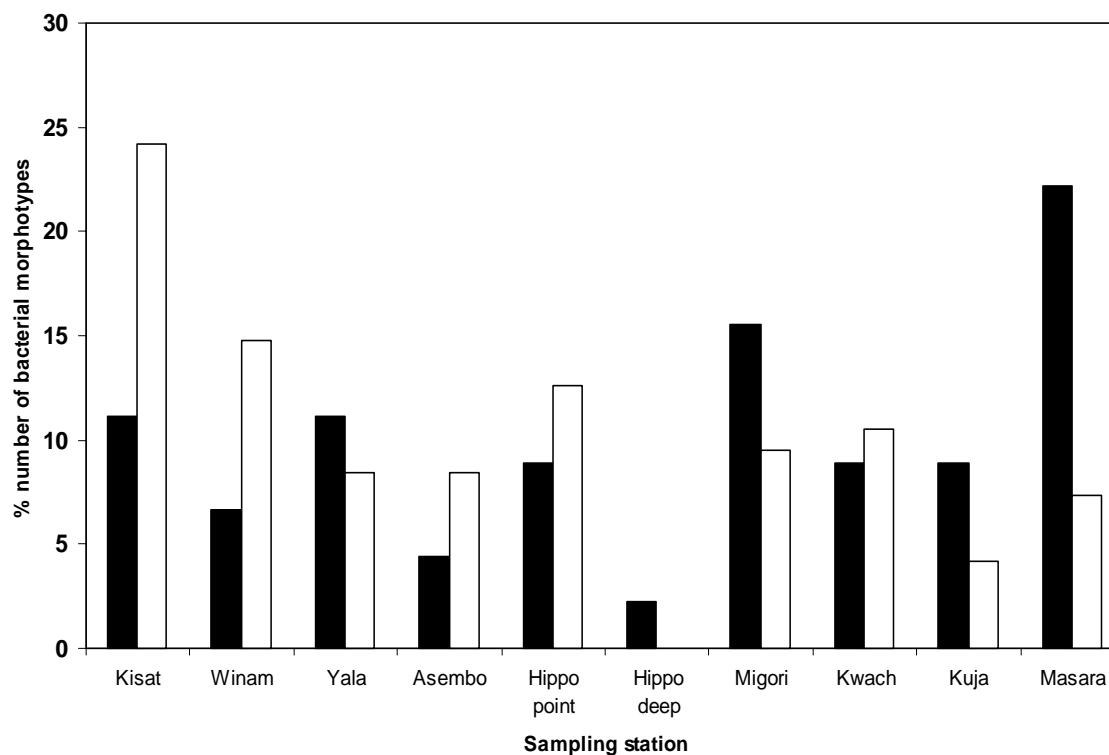W =water; S = sediment; nd = not determined

Figure 2. Diversity of bacterial morphotypes (% number) in different sampling sites around Lake Victoria sampled in the dry (■) and rainy (□) seasons
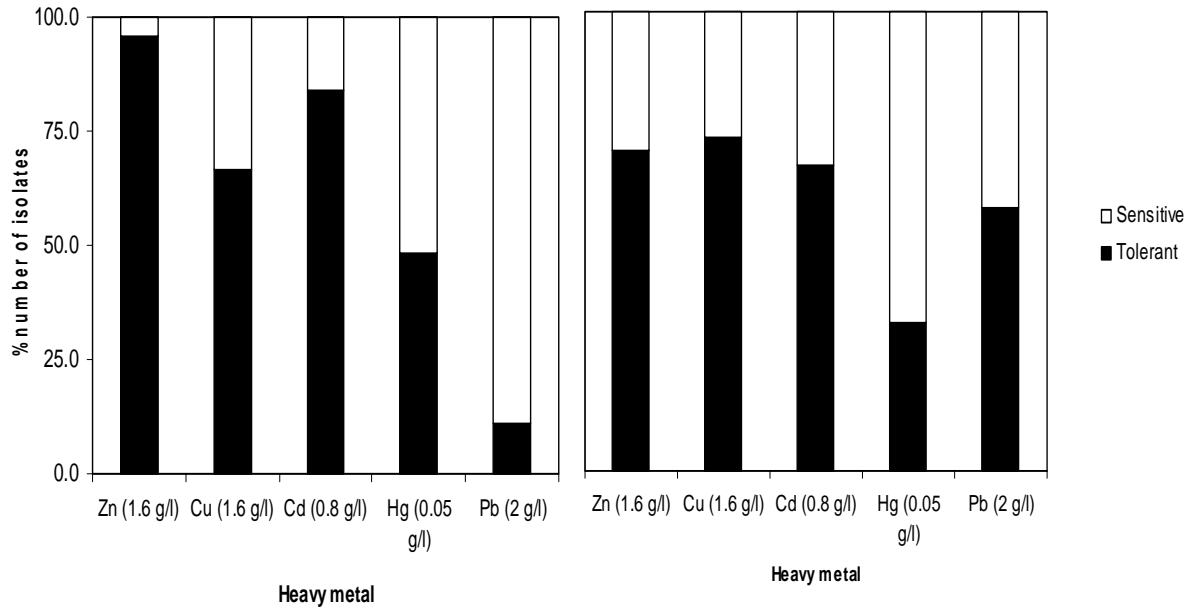
Figure 3. Heavy metal tolerance by gram-negative bacteria isolated in the dry and rainy seasons
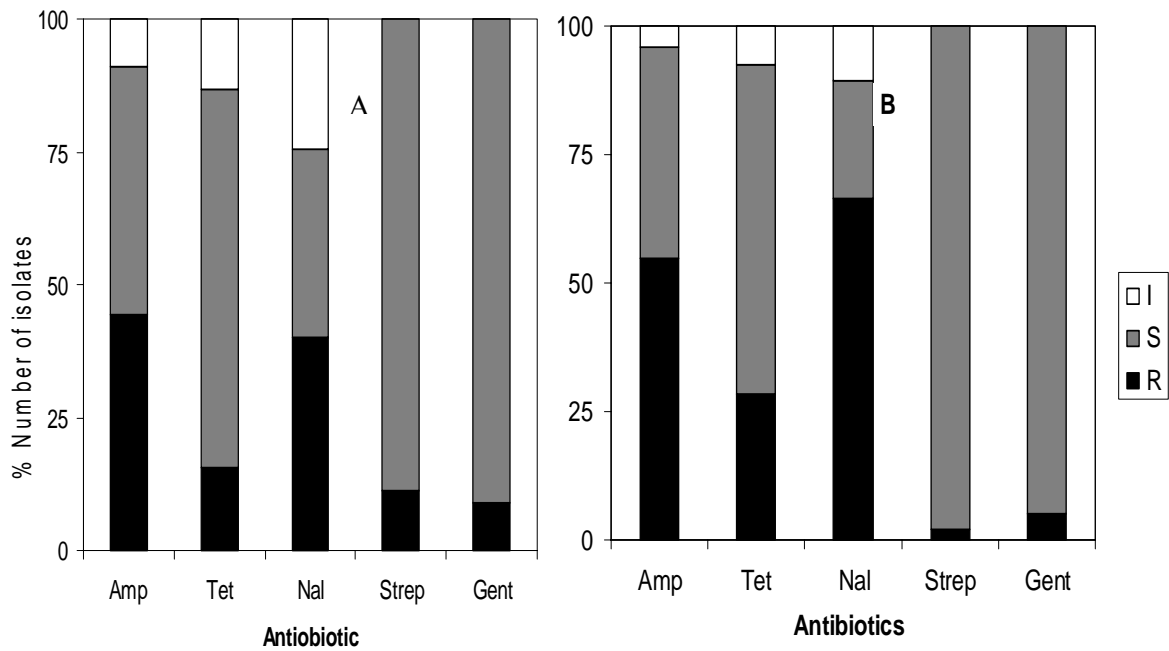

Figure 4. Antibiotic resistance patterns by gram-negative bacteria isolated in the dry (A) and rainy (B) seasons. Legend: I = Intermediate Resistance; S= Sensitive; R = Resistant

Table 2. Percentage (%) number of isolates from Lake Victoria showing different resistance patterns to two or more drugs.

	Dry Season (n = 95)	Wet Season (n =45)
Amp/Nal	12.6	8.9
Amp/Tet	5.3	4.4
Nal/Strep	0.0	2.2
Amp/Tet/Nal	20.0	0.0
Amp/Nal/Strep	0.0	2.2
Tet/Nal/Strep	0.0	2.2
Amp/Tet/Gent	0.0	2.2
Amp/Nal/Gent	2.1	0.0
Tet/Nal/Gent	0.0	2.2
Amp/Tet/Nal/Gent	0.0	4.4
Amp/Nal/Strep/Gent	1.1	0.0
Amp/Tet/Nal/Strep/ Gent	1.1	2.2

DISCUSSIONS

This study has shown that fecal contamination still poses a problem for Lake Victoria, the fish industry and the health of users of the resources in lake. During sampling in the two seasons *E. coli* which is widely accepted as an indicator of fecal contamination was isolated from largely all sampling points in the two seasons, except the Deep Hippo Point. Discussion with officials from the Municipal council of Kisumu indicated that although there are two sewage treatment plants serving Kisumu town, none is functioning, and in fact River Kasat into which allegedly treated water is discharged is virtually an open sewer flowing into the lake. Previous studies have shown fecal contamination from sewage flowing into the lake alongside heavy metal pollutions from industry and artisan gold mines as major threats facing the lake ecosystem (Kayombo and Jorgensen 2006). These problems have previously led to the suspension of fish exports to Europe.

Some of the isolates obtained in this study were tolerant to Zn^{2+}, Cu^{2+}, Cd^{2+} and a few to, Hg^{2+} or Pb^{2+}, and these were also resistant to ampicillin and nalidixic acid. Metal tolerant and antibiotic resistant bacteria have previously been isolated from environmental samples (Allen et al 1977, Baya et al 1986). Heavy metal tolerance has been implicated in increase in antibiotic resistance, through indirect selection (McAurthur and Tuckfield 2000). Grabow et al. (1974) reviewed the public health implications of drug-resistant coliforms in water supplies, and suggested that the prevalence of these drug-resistant bacteria requires reevaluation of water quality standards as well as more advanced purification of sewage prior to discharge into the environment. The ability of various gram-negative bacteria to produce disease is well known (McCabe et al 1972), and the ability of resistance factor-containing (R+) bacteria, particularly *E. coli*, to transfer drug resistance is similarly well known (Richmond 1972). The genes that control both heavy metal tolerance and antibiotic resistance are plasmid encoded and are likely to be transmitted to clinical isolates (Baya et al 1986). A number of the isolates obtained in this study were found to be resistant to more than 3 antibiotics indicating a real danger of increased incidences of multi-drug resistance among clinical isolates. These pose the real danger of increased cost of treatment of diseases in the local communities, which would otherwise be easy to treat.

REFERENCES

Al-Ghazali, M.R., Jazrawi, S.F. and Al-Doori, Z.A. 1988. Antibiotic resistance among pollution indicator bacteria isolated from Al-Khair river, Baghdad. *Water Research* 22: 641-644.

Al-Jebouri, M.M. 1985. A note on antibiotic resistance in the bacterial flora of raw sewage and sewage-polluted river Tigris in Mosul, Iraq. *Journal of Applied Bacteriology* 58: 401-405.

Baya A.M., Brayton P.R., Brown V.L., Grimes t D.J., Russek-Cohen E., and R. R. Colwell (1986) Coincident Plasmids and Antimicrobial Resistance in Marine Bacteria Isolated From Polluted and Unpolluted Atlantic Ocean Samples. *Applied and Environmental Microbiology* 51:1285-1292

Bhattacherjee, J.W., Pathak, S.P. and Gaur, A. 1988. Antibiotic resistance and metal tolerance of coliform bacteria isolated from Gomati River water at Lucknow city. *Journal of General and Applied Microbiology* 34:391-399.

Butcher, B.G. Deane, S.M. and Rawlings, D.E. 2000. The Chromosomal Arsenic Resistance Genes of *Thiobacillus ferrooxidans* Have an Unusual Arrangement and confer Increased Arsenic and Antimony Resistance to *Escherichia coli. Applied and Environmental Microbiology*. 66:1826-1833.

De Rore, H., Demolder, K., De Wilde, K., Top, E., Houwne, F. and Verstraete, W. 1994a Transfer of the catabolic plasmid RP4: Tn4371 to indigenous soil bacteria and its effect on respiration and biphenyl breakdown. FEMS Microbial Ecology 15:71-77.

De Rore, H., Top, E., Houwne, F. and Mergeay, M. and Verstraete, W. 1994b. Evolution of heavy metal resistant transconjugants in a soil environment with a concomitant selective pressure. FEMS Microbial and Ecology 14:263-273.

Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P. and Quentin, C. 2000. Impact of an Urban Effluent on Antibiotic Resistance of Riverine *Enterobacterianceae* and *Aeromonas spp*. Applied and Environmental Microbiology 66:125-132.

Grabow, W.O.K., Prozesky, O.W. and L.S. Smith. 1974. Drug resistant coliforms call for review of water quality standards. *Water Research* 8:19.

Harnandez, A., Mellado R.P. and J.L. Martinez 1998 Metal Accumulation and Vanadium-induced Multidrug Resiatnce by Environmental Isolates of Escherichia hermannii and Enterobactre cloacae. Applied and Environmental Microbiology 64: 4317–4320

Ikingura, J.R. 1994 Monitoring of mercury and other metal pollution in the Lake Victoria Goldfields, Tanzania. Technical report submitted to NEMC, 47p.

Ikingura, J.R. 1997 Mercury Pollution Due to Small Scale Gold Mining in Tanzania Goldfields. In: Landner, L (edt)1997: Environment and Mining in Eastern and southern Africa. CM Gruppen AB, Bromma, Sweden; 143–158 pp.

Ikingura, J.R. and Mutakyahwa, M.K.D 1995 Sources of Mercury Contamination and Exposure in Tanzania. In: Landner, L (edt).1995. Environment and Mining in Eastern and southern Africa. Ekonomi-Print AB, Sweden; 116– 138 pp.

Kadlec, H. R. and Knight, R. L. 1996. Treatment wetlands. Lewis Boka Raton, New York, 893 p.

Kahatano, J.M. and Mnali, S.R. 1995. Heavy Metal Contamination Due to Artisanal Gold Mining in the Tanzania Lake Victoria Gold Fields. In Landner, L (edt).1995. Environment and Mining in Eastern and southern Africa. Ekonomi-Print AB, Sweden; 66 – 81 pp.

Kayombo S. and Jorgensen S.E., 2006 http://www.ilec.or.jp/lbmi2/reports/27_Lake_Victoria_2 7February2006.pdf

Kivaisi, A. K. (2001). The potential for constructed wetlands for wastewater treatment and reuse in developing countries: A review. Ecological Engineering 16: 545 – 560 pp.

McArthur J. V. and R. C. Tuckfield (2000) Spatial Patterns in Antibiotic Resistance among Stream Bacteria: Effects of Industrial Pollution. *Applied and Environmental Microbiology* 66:3722–3726

McCabe, W. R., B. E. Kreger, and M. Johns. 1972. Type-specific and cross-reactive antibodies in gram-negative bacteremia. *New England Journal of Medicine* 287:261-267.

Mnali, S.R. 1997 Physical Environmental Pollution Resulting from Artisanal Gold Mining in the Lake Victoria Gold Fields, Tanzania. In: Landner, L (edt).1997. Environment and Mining in Eastern and southern Africa. CM Gruppen AB, Bromma, Sweden, 114–124 pp.

Newby, D.T., Josephson, K.L. and Pepper, I.L. 2000. Detection and Characterization of antibiotic resistance among various bacterial populations in a tropical river. *General and Applied Microbiology* 39:47-56.

Ogola, J.S., Mitullah, W.V., Omulo M.A. 2002. Impact of gold mining on the environment and human health: A case study in the Migori Gold Belt, Kenya. *Environment Geochemistry and Health* 24:141-158.

Pathak, S.P.Bhattacherjee, J.W. and Ray, P.K. 1993. Seasonal variation in survival and

Plasmid pJP4 Transfer to Indigenous Soil Bacteria. *Applied and Environmental Microbiology* 66:290-296.

Richards, J.W., Krumholz, G.D., Chval, M.S. and Tisa L.S. 2002. Heavy metal Resistance Patterns of *Frankia* Strains. *Applied and Environmental Microbiology* 68:923-927.

Richmond, M. H. 1972. Some environmental consequences of the use of antibiotics: or "what goes up must come down." *Journal of Applied Bacteriology* 35:155-176.

Xiong, A and Jayaswal, R.K. 1998. Molecular Characterization of a Chromosomal Determinant conferring Resistance to Zinc and Cobalt Ions in *Staphylococcus aureus. Journal of Bacteriology* 180:4024-402

Screening of Local *Bacillus thuringiensis* Isolates for Toxicity to *Chilo partellus, Sesamia calamistis* and *Busseola fusca* in Kenya

Wang'ondu[1] VW, JHP Kahindi[2], NK Olembo[3] and JO Ochanda.[3*]
[1]School of Biological Sciences, University of Nairobi, vwwangondu@yahoo.com
[2] School of Arts and Sciences, United States International University, P.O. Box 14634, 00800, Nairobi, jkahindi@yahoo.com
[3]Department of Biochemistry, University of Nairobi, P. O. Box 30197, 00100, Nairobi
[3 *] Corresponding author, jochanda@uonbi.ac.ke

ABSTRACT

Stem borers are a major source of pre-harvest maize crop losses in Kenya and many Sub-Saharan African countries. This menace needs to be addressed if food security is to be realized in this region. Seven local isolates of *Bacillus thuringiensis* (Bt) strains were isolated from soils collected from Kakamega and Machakos districts in Kenya. They were screened for toxicity against 1st and 2nd instar larvae of *Chilo partellus, Sesamia calamistis* and *Busseola fusca* through laboratory bioassays on artificial and natural diets. On farm Bt toxin potency trials were carried out only in Machakos using isolate 1M which was isolated from the area. The various isolates showed differences in their toxicity to the three stem borers. Isolates 1M and VM-10 (from Machakos district) were found to be the most potent against *C. partellus* with larval mortalities of 100 % within 72 h. Their LD$_{50}$ values were 0.004 mg/ml and 0.04 mg/ml respectively. The most toxic isolates against *S. calamistis* were, 44M, VM-10 and 1M, with larval mortalities of 73%, 64% and 62% respectively after 72h at a concentration of 8.6 mg/ml through artificial diet bioassays on 1st instar larvae. Isolates 44M and K10-2 showed high toxicity against *B. fusca* with larval mortalities of 20% by artificial diet bioassays and 44% by maize leaf bioassays respectively. Leaf disk bioassays with all the insect species showed higher larval mortalities than those done with the artificial diet bioassays indicating the larval preference of natural diet. However leaf disk bioassays with *B. fusca* recorded higher larval mortalities with sorghum than maize leaves. Field trial results obtained from Machakos district using a biopesticide made from isolate 1M indicated that it was highly effective in stem borer control.

Key Words: *Bacillus thuringiensis*; bioassays; *B. fusca; C. partellus*; *S. calamistis*; insect larvae; mortality; toxicity.

INTRODUCTION

Africa's food deficit is largely due to pre-and post-harvest crop losses, especially for graminaceous crops such as maize, rice, wheat and sorghum (Brownbridge, 1991). Other causes of decreased food production include drought and land degradation. However a major threat to increased food production is damage to crops by insect pests both in pre-and post harvest periods. These are the major constraints to efficient cereal production in developing countries.

Among lepidopteran pests, the spotted stem borer (*Chilo partellus)* (Swinhoe) and the maize stem borer *(Busseola fusca)* (Fuller) rank as the most important stem borer pests of sorghum and maize in Africa (Sithole, 1989); (Seshu Reddy, 1989). They are predominant borer species of economic importance in Eastern Africa and Southeast Asia (Brownbridge, 1991). Locally, *C. partellus* is an

introduced species, which has spread in both maize and sorghum growing areas below 1500m (Zhou *et al*., 2001b; Songa *et al*., 2002b). *Sesamia calamistis* is a native species occurring in areas of up to 2400m, whereas *B. fusca* is found in all areas of up to 2600m but not in the lowland tropics (Hilbeck and Andow, 2004). The pests feed exclusively on graminaceous plants whereby heavy infestations may result in severe crop losses and hunger, given that maize and sorghum are staple foods in Kenya and mostly grown by subsistence farming. Infestation levels and yield losses caused by *C. partellus* have not been quantified in most countries in the sub region. Estimates reported in publications have been based on visual evaluations of the infestations (Sithole, 1989a). However farmers in Kenya estimate that the stem borers cause significant yield losses in their maize crops (Hassan *et al*., 1998b). In Nigeria, the presence of one or two larvae of *B. fusca* per plant reduced the yield by as much as 25% (Usua, 1968). Control

measures have largely been based on chemical pesticides (insecticides). These broad-spectrum chemicals have been used abundantly in the containment or eradication of pests. This has been accompanied by a host of problems such as, development of resistance by the pests, destruction of natural enemies, environmental degradation, and toxicity to human beings and above all they are not affordable to the resource poor farmers who form the bulk of the food producers in the country.

Recent promising progress in agricultural entomology and microbiology demonstrates that there is a considerable unexploited potential (Dulmage, 1993). The microbial pesticides based on *Bacillus thuringiensis* (*Bt*) can in an integrated control programme be used rapidly and efficiently to destroy insect pests. A number of advantages are associated with the use of *Bt*, such as non-mammalian toxicity, target specificity and safety to non-target organisms, complete biodegradability into non-toxic products and relatively low costs of production through fermentation (Kirschbaum, 1985; Lisansky, 1989). More recent developments of transgenic plants expressing *Bt* endotoxins are being developed by Insect Resistant Maize for Africa (IRMA) program.

Kakamega and Machakos districts are major maize growing areas in Kenya mainly by subsistence farming. Most farmers experience heavy pre-harvest crop losses as result of stem borer infestations. Continued cultivation of maize has led to the increase in the number of stem borers. This study therefore undertook to isolate local *B. thuringiensis* isolates from maize growing areas of kakamega and Machakos (Wang'ondu *et al*., 2003) and screen them for potential toxicity to stem borers. Local isolates could potentially pose better characteristics such as greater field persistence and toxicity at a higher temperature ranges (Brownbridge, 1991). The potent isolates potentially can be incorporated into a pest control programme thus in the long run offer a solution and provide a cheaper alternative to the increasing threat to food production. In this paper we report the potency of the local *Bt* isolates to *C. partellus, B. fusca* and *S. calamistis*.

MATERIALS AND METHODS

Bt- isolates
The seven isolates (1M, 44M, V14-M, V24-M, VM-10, K10-2 and 12F-K) were isolated from soil samples from Kakamega and Machakos Districts in Kenya (Wang'ondu *et al*., 2003). Two other isolates were also tested alongside our isolates. Thuricide® a commercial product obtained from the market and "MJ 99 2" from Insect Rearing and Quarantine Unit at the International Centre of Insect Physiology and Ecology (ICIPE).

Insects and artificial diet
Neonate larvae of the test organisms and their artificial diet were purchased from the Insectary Unit at ICIPE. Insects were reared on a Lepidopteran artificial diet. Neonate (1st instar) and 2nd instar larval stages of *C. partellus*, *S. calamistis* and *B. fusca* were used for the toxicity bioassays. However due to the high cost of the 2nd instar larvae, only a few bioassays were carried out with this larval stage.

Growth of isolates for bioassays
Sporulated cultures of *B. thuringiensis* isolates were used to inoculate 50 ml of Nutrient Broth (Oxoid) (Lab-Lemco' powder 1.0 g; Yeast Extract 2.0g; Peptone 5.0g; Sodium Chloride 5.0 g per litre) in 250 ml fluted Erlenmeyer flask. The inoculated flasks were incubated on a rotary shaker for 96 h at 30º C at 200 r.p.m. At the end of the incubation period, the spore-crystal complex was harvested by centrifugation at 4000 r.p.m. for 10 min. The supernatant was discarded and the pellet washed three times by centrifugation in sterile 0.85% saline and suspended in 5 ml sterile saline water (0.85%) and stored at –20º C.

Laboratory toxicity (artificial diet) bioassays
Bioassays were performed using spore-crystal suspension on 1 day-old neonate larvae of the three insect pests. Molten artificial diet, which was cooled to 50 ºC before incorporation of the *B. thuringiensis* spore-crystal suspension (which, contains the delta-endotoxin) of known concentration due to the heat sensitive nature of the *Bt* toxin. 1st and 2nd instar larvae were used for bioassays with *C. partellus*, whereas 1st instar larvae were used for *B. fusca* and *S. calamistis* assays. Thuricide® and "MJ 99 2" were only tested against *C. partellus* 1st instar larvae. Protein concentration of spore-crystal complex of the different isolates was determined by the BSA method, which reflected the toxin concentration. The suspensions were then added to the diet to produce desired final concentrations. The diet was then dispensed into 20 ml tubes and allowed to cool to room temperature. One insect larva was introduced per tube and incubated at 25±2ºC. Appropriate positive and negative controls were carried out at the same time. All assays were replicated three times, each with 15 larvae. Data (larval mortality) was recorded after 24 h for 5 days (120 h), which was measured in terms of immobility (unresponsive) and colour change. This process was followed for each of the *Bt* strains that were used in the study. The LD_{50} was calculated by probit analysis. Where control mortality exceeded

5% but not higher than 10%, data was kept and analyzed by probit analysis after adjusting for control mortality (Abbott's, 1925).

Leaf disk bioassays

All leaf disk bioassays were carried out with 1st instar larvae except for *B. fusca* where 2nd instar larvae was used. A concentration of 8.6 mg/ml of the *Bt* toxin from the 7 isolates was used. Sorghum leaves were only tested with *B. fusca* . Four excised maize / sorghum leaves (2 cm^2) three weeks old were immersed in a suspension of *B. thuringiensis* spore-crystal suspension of known concentration and then allowed to dry making sure all the surfaces of the leaf were covered. They were then placed in a petri dish. A piece of wet filter paper (dipped in distilled water) was placed in the dish to provide moisture. 15 larvae were introduced per dish. The dishes were then sealed with two straps of stretched parafilm and incubated at 25±2ºC. In the control dish untreated leaf disks were used. Larval mortality was recorded after every 24 hrs for 5 days. The experiment was done in triplicates.

On farm trials of *Bt* toxin potency

Isolates that were found to be highly potent by leaf disk bioassay were later selected for the on farm trials in their areas of isolation. This was to test whether the same toxicity level shown by the isolates can be demonstrated in the natural field environment. In this paper we report on farm trials for Machakos District. Large scale production of *B. thuringiensis* toxin based on isolate 1M was carried out in the lab using the same method for the preparation of the toxin for the laboratory bioassays and used for the trials in Machakos district, Mwala division, Masii location. Four farmers who owned the farms from which soil samples had been collected for *Bt* isolation participated in the project. The farmers prepared the farms and planted their maize crop in normal planting seasons. It is from these farm sites that a plot of 10mx10m was identified for the on farm *Bt* toxin potency trials. A control plot measuring the same size was also identified 100 metres from the treatment plot site. Initial spraying of the *Bt* toxin was done three weeks after planting using a Knapsack sprayer and another application was done after two weeks. This is because stem borer infestation occurs 2-3 weeks after planting, and this is when major damage to the crop is realised. Monitoring of the fields was done weekly up to the time of harvest. Individual analysis of each farm was done, as the farms were far apart. Presence of stem borers on the crop was noted by recording the extent of damage (holes) on the maize leaves in the early stages of infestation and also by the presence

of dead hearts (undeveloped maize cobs) in the later stages.

RESULTS

Artificial diet bioassays

*Chilo partellus***:** Results from the artificial diet bioassays with 1st instar larvae of *C. partellus* indicated that isolates 1M and VM-10 at a concentration of 8.6 mg/ml isolates with a larval mortality of 100% after 72 hrs (Fig. 1a). However all the isolates recorded a larval mortality of 70% and above, hence indicating their potency against the stem borer. All the isolates were also equally potent against 2nd instar larval stage and recorded larval mortalities above 70% (Fig. 2). The results indicated that both larval stages were highly susceptible to the toxin of the *B. thuringiensis* isolates, however higher mortalities were observed for the 2nd instar larvae. All the isolates caused higher larval mortalities than the commercial *Bt* preparations of Thuricide® and "MJ 99 2", which recorded larval mortalities of 69% and 58% respectively (Fig. 1a).

*Sesamia calamistis***:** Lower larval mortalities were recorded for artificial diet bioassays on all isolates against *S. calamistis* than for *C. partellus* (Fig 1a & b). The highest larval mortality was 73% by isolate 44M on 1st instar larvae after 72h at a concentration of 8.6mg/ml (Fig. 1b). The isolate K10-2 had the lowest potency against *S. calamistis*. None of the tests were carried out with 2nd instar larval stage.

*Busseola fusca***:** The *B. fusca* test organism recorded the lowest larval mortalities of the three species tested on the artificial diet. The highest larval mortality recorded was 20% by isolate 44M in 72h on 1st instar larvae (Fig. 1c). The lowest mortality rate was recorded using isolate V24-M.

Leaf disk bioassays

*C. partellus***:** Maize leaf bioassays on *C. partellus* showed high larval mortalities in comparison with artificial diet assays, with all the isolates causing 100% larval mortality in 48h after exposure to 8.6mg/ml concentration of δ-endotoxin of *Bt* isolates (Fig. 3a).

S. calamistis: Larval mortality for *S. calamistis* was not significantly higher than that for artificial diet bioassays. However, isolates V14-M and V24-M showed larval mortalities of 72% and 73% respectively in 72h (Fig 3b). The lowest mortality rate of 40% was recorded using isolate K10-2 from Kakamega.
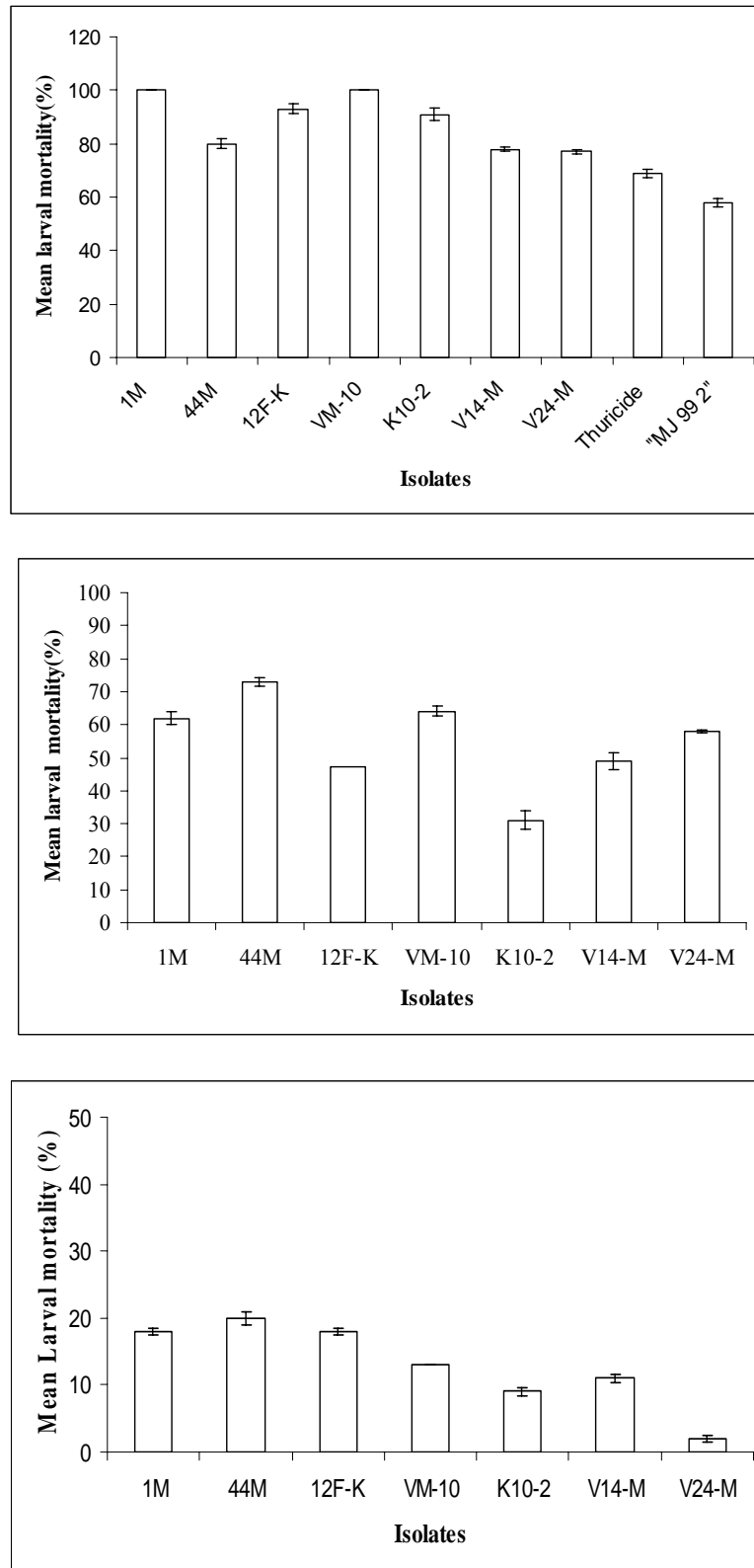
Fig. 1 (a) Larval mortality of 1st instar larvae of *C. partellus* **(b)** *S. calamistis* and **(c)** *B. fusca* after exposure to 8.6-mg/ml δ-endotoxin of different *B.t* isolates. Incubation was at 25 ±2 °C for 72 h.
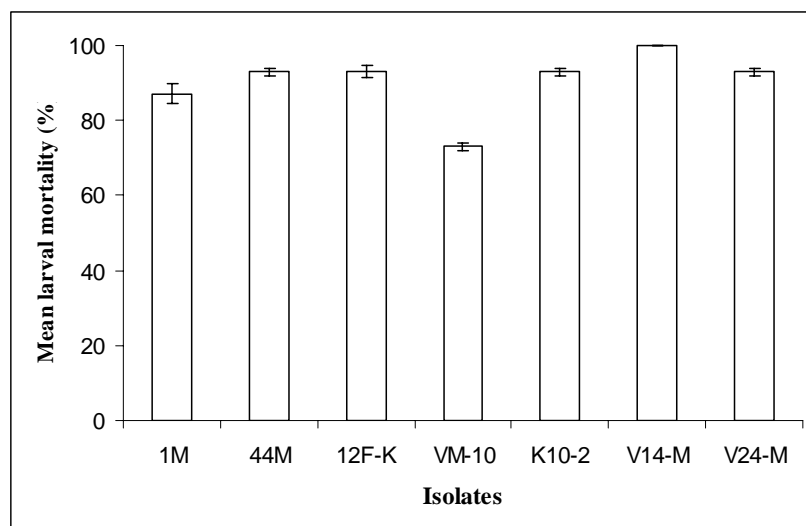
Fig. 2: Larval mortality of 2nd instar larvae of *C. partellus* after exposure to 8.6-mg/ml δ-endotoxin of different *B.t* isolates through artificial diet bioassays. They were incubated at 25 ±2 °C for 72 h.

B. fusca: Percentage mortality levels with maize leaf disks were not significantly different from those observed with artificial diet bioassays. However they were lower than those for other test organisms. Isolate K10-2 recorded the highest larval mortality of 44% in 72h (Fig. 3c). All the *B t* isolates caused higher larval mortalities on assays with sorghum leaves than with maize leaves, with isolate K10-2 causing a larval mortality of 87% at 72 hrs (Fig.3c). This also indicated the *B. fusca* larvae preference for sorghum to maize leaves. Mortality of 1st instar larvae assayed on three weeks old sorghum leaves and treated with varying concentrations of the toxin showed that, increase in time resulted in increased larval mortality within the same concentration. However, increase in toxin concentration did not automatically result in an increase in larval mortality (Fig. 4). Toxin concentrations of 8.6 and 17.2 mg/ml caused lower mortality levels than 0.86 mg/ml at 48hrs. At 24hrs 17.2 mg/ml caused a lower mortality compared to 8.6 mg/ml.

Field trials for *Bt* toxin potency
On farm trials results obtained from Machakos District using *B. thuringiensis* biopesticide based on isolate 1M, showed that the number of dead hearts which form after an attack by the stem borers were very minimal in the treated plots than in the control plots within the different farms (Fig.5). None of the dead hearts were observed in the treated plot on farm 1. In farm 1 there was 100% control of the stem borer *C. partellus* that is common in the area. All control plots recorded higher number of dead hearts in all the farms.

DISCUSSION
All the *B. thuringiensis* isolates were highly toxic to *C. partellus* larvae. However there were variations among the isolates with regard to their larval toxicity. This implies that *B. thuringiensis* strains exhibit specificity. All the isolates indicated higher toxicity levels than the commercial product Thuricide® and " MJ 99 2 " and this relates with the findings of Brownbridge, 1991 whereby he found some of the local *Bt* isolates to be more toxic than the exotic ones. Many of the well-characterized *Bt* strains have been isolated from temperate zones and may not be as well suited for use in pest management programmes in the tropics as local isolates, which may possess useful attributes such as greater field persistence at high temperatures.

Percentage larval mortalities of the 1st and 2nd instar stages of *C. partellus* did not vary significantly. The two instars were highly susceptible to the *B. thuringiensis* toxin of the different isolates. The result can be explained by the fact that they are the active feeding stages of the larvae. These results are related to the findings of Karamanlidou *et al.*, (1991). He reported that mortality levels of *Drosophila oleae* caused by the toxin of different isolates of *B. thuringiensis* are always higher if the experiments are carried with larvae rather than the adults. Young larvae (1st and 2nd instars) than mature larvae succumbed to infection earlier than the mature larvae. Older larvae (3rd and 4th instars) of these target pests feed minimally as they are ready to pupate, compared to 1st and 2nd instars that feed more actively. They should thus be targeted during a pest control program.
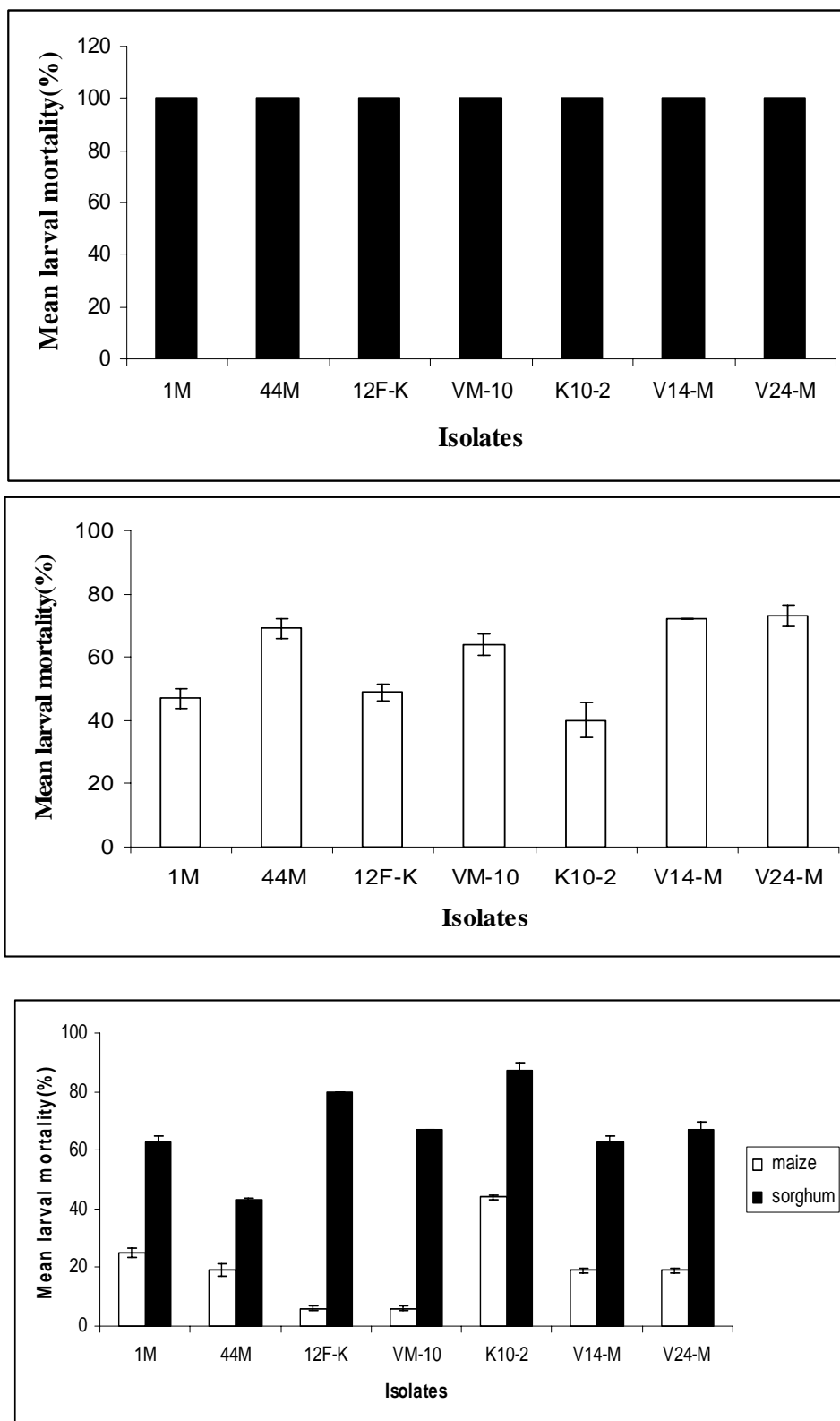
Fig. 3 Larval mortality of 1st instar larvae of **(a)** *C. partellus,* **(b)** *S. calamistis* and **(c)** *B. fusca* after exposure to 8.6-mg/ml δ-endotoxin of different *B.t* isolates through leaf disk bioassays with maize leaves. They were incubated at 25 ±2 °C for 48 h.
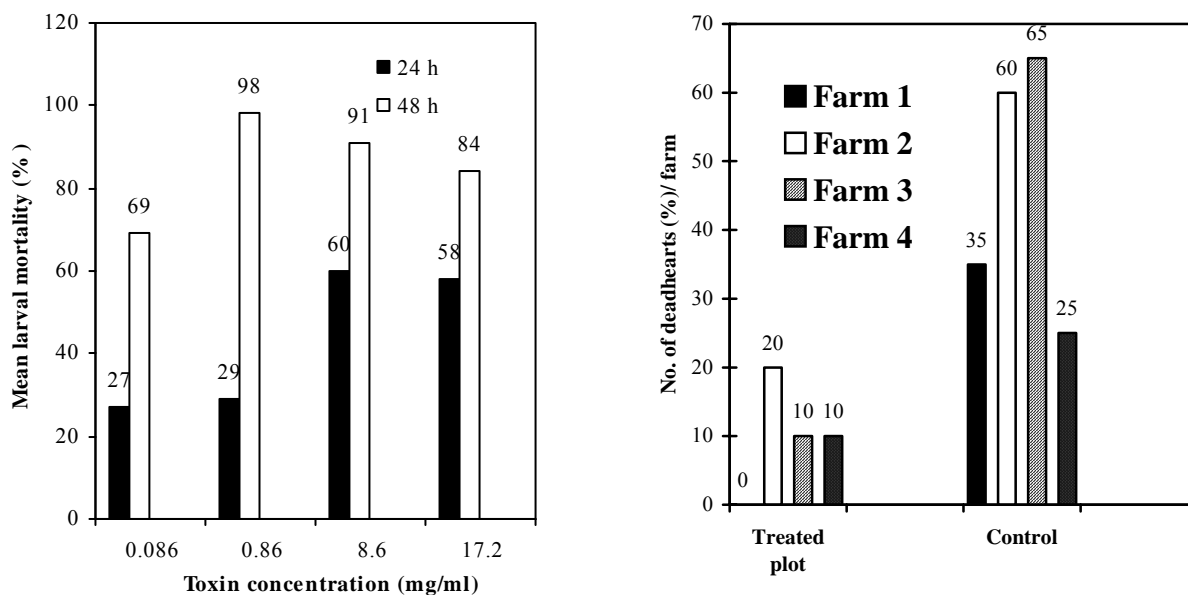
Fig 4. **(a)** Percentage mortality of 1st instar larvae of *B. fusca* larvae fed on sorghum leaves treated with varying concentrations of *B. thuringiensis* toxin of isolate **1M** by leaf disk bioassay method. Incubated was at 25 ±2 °C for 48 h and **(b)** comparison of the control of borer infestation in the *B. thuringiensis* treated and control plots by the number of dead hearts.

The data obtained clearly indicates the strong potential of using *B. thuringiensis* for the control of cereal pests.

Percentage larval mortalities were higher for *C. partellus* larvae followed by *S. calamistis* and *B. fusca* in decreasing order. This indicates the fact that *B. thuringiensis* has a narrow range of activity and is highly specific. From this study it was observed that different *Bt.* isolates have different levels of toxicity, even for the same pest species under test. This was consistent with findings of (Brownbridge 1992). All the *B. thuringiensis* isolates showed marked differences in their levels of activity against the three pest species (*C. partellus*, *B. fusca* and *S. calamistis*).

Differences in the levels of toxicity to the three pest species demonstrated the need for screening a large number of *B. thuringiensis* strains when considering their use in a microbial control programme. It is also important to screen the *B. thuringiensis* isolates, for their potency against a wide range of insect pests, since one isolate that is not toxic to a certain pest may be highly toxic to another. This calls for screening the isolates against many Lepidopteran, Dipteran and Coleopteran insect pests. This is because *B t* toxin is highly specific and is a narrow spectrum biopesticide. Some preliminary tests done with the isolates showed that they are toxic to *Helicoverpa armigera* (a legume pod-borer). Such inter-varietal differences have been observed and reported in a

number of other studies (Amonkar *et al.*, 1985; Jarret and Burges 1986; Padua *et al.*, 1987).

Unlike with *C. partellus* where death was observed after 24 h, death for *S. calamistis* and *B. fusca* was observed after 48 h. Larval mortalities for *B. fusca* were much lower than for the other test species. Cherry *et al.*, (1999) reported that the concentration required to cause 50% larval mortality was higher for *B. fusca* than that for other species. This could also be explained by the fact that, *B. thuringiensis* exhibits anti-feedant effects, that may inhibit full ingestion leading to variability in the amount of toxin obtained by larvae. This was also observed during the current study, where the larvae, on introduction to the tubes with treated artificial diet, located themselves on top of the tube (near the gauze) for some time during which there was minimal feeding or none.

The differences in larval mortality using the different bioassay methods indicate the diet preference of natural diet to artificial diet; hence more toxin was consumed in natural diet than in the artificial diet. Unlike in the artificial diet bioassays where the larvae took some time before they started feeding, larvae in the leaf disk bioassays started feeding immediately after introduction. This indicated that the larvae preferred their natural diet. However larvae in the treated leaves were feeding minimally as compared to the controls. The extent of damage of the leaves

was directly related to the toxicity of the isolate. There was more leaf damage in the controls than in the treated leaves. Among the isolates, most damage to the leaves was found in the leaves treated with less toxic isolates than with the more toxic isolates. All the larvae in both the treatments and the controls preferred the under side of the leaf disk.

The study indicated that increase in concentration above a certain level does not necessarily result in an increase in larval mortality. Therefore there is a particular concentration where maximum larval mortality is obtained and any further increase in toxin concentration only results in a decline in larval mortality. The preliminary field study conducted in Machakos district indicated that *B. thuringiensis* based biopesticides can be an effective way of controlling stem borers especially *C. partellus* that was encountered during the study, and which is a resident of the low altitude areas. Isolate 1M was originally isolated from the area. Isolate K10-2, which was the most potent against *B. fusca*, was isolated from Kakamega soil samples. *B. fusca* is responsible for maize damage in the high altitude regions. This gives support to the idea that local isolates may be well suited to the local environmental conditions than exotic isolates which are isolated from temperate zones which exhibit different environmental conditions. Leaf disk bioassays with *B. fusca* indicated higher larval mortalities with sorghum than maize leaves. This can partly be explained by the fact that maize contains volatile repellent molecules whereas the volatile molecules found in sorghum are attractive hence the larvae prefer feeding on sorghum than maize. The study recommends large scale production of these highly potent isolates which should be made available to the farmers at affordable rates. The isolates may also be further tested for their potency against other pests. The isolates, may also be incorporated in the on going research on *Bt*. maize since it may stand a better chance in the control of maize borers than the *Bt* biopesticides since the latter, have some constraints in that they have a quick breakdown and timing and coverage are critical as spores must be fed on to have any biocontrol activity and only small larvae are susceptible. The isolates are currently preserved at Centre for Biotechnology and Bioinformatics (CEBIB), College of Biological and Physical sciences, University of Nairobi.

REFERENCES

Amonkar, S. V., Kulkarani, U., and Anand, A. (1985). Comparative toxicity of *Bacillus thuringiensis* subspecies to *spodoptera litura* (F). Current Science 54: 473-478

Brownbridge, M. (1991). Native *Bacillus thuringiensis* Isolates for the management of Lepidopteran cereal pests. Insect Science and its Applications 12:57-61.

Brownbridge, M. and Onyango, T. (1992). Screening of exotic and locally isolated *Bacillus thuringiensis* (Berliner) strains in Kenya for toxicity of the spotted stem borer *Chilo partellus* (swinhoe). Tropical Pest Management 38: 71-81.

Cherry, A. J., Lomer, C. J., Djegul, D. and Schulthess, F. (1999). Pathogen incidence and their potential as microbial control agents in IPM of maize stem borers in West Africa. Biocontrol 44:301-327

Hassan, R. M. Onyango, R. and Rutto, J. K. (1998b) Relevance of maize research in Kenya to maize production problems perceived by farmers. In Hassan, R. M. (ed) maize technology development and transfer: a GIS Application for research planning in Kenya.CAB Internatinal, Wallingford,UK, pp. 71-88.

Hilbeck, A. and Andow, D. A. (eds) (2004) Environmental Risk Assessment of Genetically Modified Organisms: Vol. 1. Case Study of *Bt* maize in Kenya. CAB International, Wallingford, UK.

Jarret, P and burges, H. D. (1986). Isolates of *Bacillus thuringiensis* active against Mamestra brassicae and some other species: alternatives to the present commercial isolate HD-1. Biological Agriculture and Horticulture, 4:39-45.

Karamanlidou, G., Lambropoulus, A. F., Koliais, S.I., Ellar, D., and Kastritsis, C. (1991). Toxicity of *Bacillus thuringiensis* to laboratory populations of the olive fruit fly (Dacus oleae). Applied Environmental Microbiology 57: 2277-2282).

Kirschbaum J. B. (1985). Potential implication of genetic engineering and other biotechnologies to insect control. Annual Reviews Entomology 30:51-70.

Lisansky S. G. (1989). Biopesticides. Ag Biotechnolgy News and Information 1: 349-353.

Martin, P. A.W., and R. S. Travers (1989). Worldwide Abundance and Distribution of *Bacillus thuringiensis* Isolates. Applied Environmental Microbiology 55(10): 2437-2442.

Padua, L. E., Gabriel, P. P., Aizawa, K., and Ohba, M. (1982). *Bacillus thuringiensis* isolated from Philippines. Philippines Entomology 5: 199-208.

Seshu Reddy, K. V. (1989). Sorghum Stem Borers in Eastern Africa. In Proceedings of the International Workshop on Sorghum stem borers. 17-20th November, 1987. ICRISAT, India. pp 33-40.

Sithole, S. Z. (1989). Sorghum stem borers in Southern Africa. In Proceedings of the International Workshop on Sorghum stem borers. .17-20th November 1987. ICRISAT, India. pp 41-47.

Sithole, S. Z. (1989a). Maize stem bores-yield loss and damage caused by maize stem borers in Eastern, Central and Southern Africa. In crop Protection for small scale Overholt, W. A. farms in East and Central Africa-a Review (Edited by Prinsley, R. T. and Terry, P.S.) pp.72-83. Commonwealth Science Council Marlborough Houde Pall, London.

Songa, J. M., Overholt, W. A., Okello, R. O. and Mueke, J. M. (2002b) Control of lepidopteran stem borers in maize by indigenous parasitoids in semi-arid areas of eastern Kenya. Biological Agriculture and Horticulture 20, 77-90.

Usua E. J. (1968). Effect of varying populations of *Busseola fusca* larvae on growth and yield of maize. Journal of Economic Entomology 61:375-376.

Wang'ondu *et al*., (2003). Isolation and Partial characterization of *Bacillus thuringiensis* strains from Soil Samples from Kakamega and Machakos Districts in Kenya. Journal of Tropical Microbiology, **2**: 3-10.

Zhou, G., and Mochiah, M. B. (2001b) Changes in the distribution of lepidopteran maize stem borers in Kenya from the 1950s to 1990s. Insect Science and its Application 21, 395-402.

A Latex Agglutination Test for Capripoxvirus

Muinamia[1*] K, YS Binepal[3*], J Machuka[2], J Makumi[2] and R Soi[3]
[1]Department of Biochemistry, Faculty of Medicine, University of Nairobi,
P.O. Box 30197, Nairobi, Kenya
[2]Department of Biochemistry and Biotechnology, School of Pure and Applied Science, Kenyatta University, P.O. Box 43844, Nairobi, Kenya
[3]Biotechnology Center, National Agricultural Research Laboratories, Kenya Agricultural Research Institute, P. O. Box 14733, Nairobi, Kenya
*Corresponding author. P.O. Box 57811, Tel: 4440113, Fax: 4440113, E-mail: ybinepal@yahoo.com; ybinepal@nbi.ispkenya.com

ABSTRACT
The gene Q_13L coding for the *Capripoxvirus* group specific structural protein P32 was expressed in *Escherichia coli* using plasmid pGEX-2T as a fusion protein with glutathione-s-transferase and purified on glutathione sepharose affinity chromatography column. The protein was then employed for diagnosis of sheeppox, goatpox and lumpyskin disease, by a latex agglutination test (LAT) using the purified P32 antigen and guinea pig detector antiserum raised against the P32 antigen. The LAT and virus neutralization test (VNT) were used to screen one hundred livestock field sera for antibodies to *Capripoxvirus*, in comparison the LAT was simpler, rapid and 23% more sensitive than the VNT. In addition the LAT was found to be specific for *Capripoxvirus* because it did not pick antibodies to *Orthopoxvirus* and *Parapoxvirus*. The LA test can be taken for a simple and quick diagnostic tool for primary screening of *Carpripoxvirus* infection and will reduce the reliance of diagnostic laboratories on tissue culture facilities.

Key words: Carpripox, latex agglutination test, attachment gene

INTRODUCTION

Sheeppox (SP), goatpox (GP) & lumpyskin disease (LSD) are pox diseases of sheep, goats and cattle respectively (Kitching, 2003). These diseases are caused by poxviruses of the genus *Capripoxvirus* (Van, 2005). SP and GP are prevalent in most areas of Africa, Middle East, Indian sub-continent and Southwest Asia, while LSD has slowly spread throughout much of Africa (OIE, 2002).

Capripoxviruses cause severe economic loses in countries where they are endemic (Heine, 1999), mainly due to decreased body weight, decreased milk production, abortions, low quality of hides and infertility in bulls (Subba, 1984). Clinical symptoms are characterized by pyrexia, generalized skin, internal pox lesions and enlargement of superficial lymph nodes. The morbidity and mortality of SP & GP is 80% to 100% and 50%, respectively, while that of LSD is 3% to 85% and 2-20%, respectively (OIE, 2005). *Capripoxviruses* infection in sheep and goats is transmitted mainly by natural inhalation of aerosols and close contact between animals (Carn, 1995), while that of LSD mainly occurs as a result of insect bites (OIE, 2004).

Early diagnosis of *Capripoxvirus* infection is of paramount importance in the control of the disease since no cure is available (OIE, 2002). This allows rapid control measures to be instigated to limit loses such as quarantine, ring vaccination and slaughter (Carn, 1993). *Capripoxvirus* infection is usually diagnosed by clinical signs but its confirmation is made by serological tests (Rao, 2000), such as virus neutralization test (VNT) (OIE, 2005; Prescausta, 1979), enzyme-linked immunosorbent assay (ELISA) (Carn, 1995; Rao,

1997) or counter immunoelectrophoresis (CIE) (OIE, 2005; Sharma, 1988). These facilities are limited to a small number of central laboratories and transportation of samples is difficult. There is a dire need for a diagnostic test to confirm *Capripoxvirus* infection that is rapid, simple, affordable and one that can possibly be performed under field conditions.

Virus neutralization test (VNT) is the only widely used serological test for antibodies against *Capripoxvirus*, the VNTs are used in many laboratories but are time consuming and require high standard facilities and qualified technical personnel. Identification of infected cells may be difficult, and toxic effects can mimic cytopathic effect (CPE) caused by virus multiplication (Carn 1994). The VNT plates must be incubated for 9-12 days before the end point can be determined, requiring good aseptic technique, an incubator and a reliable power source (Prescauta, 1979). This implies that other tests need to be adopted especially for developing nations that do not have access to such high standard facilities.

Agar gel immunodiffusion test (AGID) (Davies, 1976) and indirect fluorescent antibody technique (IFAT) (Subba, 1984) have been used for detection of *Carpripoxvirus* precipitating antigens and antibodies respectively. These tests are inadequate in that AGID lacks specificity due to cross reaction with *Parapoxvirus* precipitating antigens (Kitching, 1986a) while IFAT does show low level cross reaction with *Cowpoxvirus* sera (Davies 1982). On the other hand electron microscopy cannot distinguish *Capripoxvirus* from *Cowpoxvirus* and *Orthopoxvirus* (Kitching, 1986b). These tests are

therefore unsuitable for detection of *Capripoxvirus* infection because they can give false positive results.

SP, GP and LSD are serologically indistinguishable, having group specific protein P32 antigen, which is a major component of the external membrane (Hossamani, 2004). The P32 protein was shown by immunoblotting to contain an important *Capripoxvirus* specific antigenic determinant, the kinetics of antibody response to the structural proteins of the *Capripoxvirus* reference strain KS-1 showed that the P32 protein produced an antibody response in advance and in excess of that produced by other structural proteins (Chand, 1992). The viral genes that encode for the P32 protein can be expressed within a bacterial system, thus eliminating the need for tissue culture and allowing for sufficient amounts of recombinant protein to be produced readily and economically. Recombinant proteins can be used directly in immunoassays and avoid the risk of spreading the virus in the laboratory.

The previous serological tests that are tissue culture dependant were expensive and unreliable (Kitching, 1992). There results were delayed up to two weeks for the virus cytopathic effect to develop (Plowright, 1958). In addition *Capripoxvirus* infection is cell mediated and low level specific antibodies produced after infection made interpretation difficult (Srivastava, 1980). There was therefore a requirement for a specific and sensitive test to detect antibody to *Capripoxvirus* that is not dependant on tissue culture.

MATERIALS AND METHODS
Plasmid pVC-1
Plasmid pVC-1 was donated by Institute of Animal Health (Fulbright), courtesy of Dr. V.N.Carn (University of Reading, England). The plasmid pVC-1 was derived by insertion of *Capripoxvirus* gene Q_13L into the unique cleavage site in the pGEX-2T polylinker region. *Capripoxvirus* gene Q_13L, encoding protein P32, was cloned into the BamHI and EcoRI site in the commercial expression vector pGEX-2T (Pharmacia, Uppsala. Sweden). The tac promoter allows controlled expression of the fusion protein by induction with isopropyl-beta-D-thiogalactopyranoside (IPTG). The protein is produced as a fusion protein glutathione-S-transferase- P32 (GST-P32) with a molecular weight of 36 KDa.

Transformation of E.coli
Competent *E.coli* were prepared and transformed with plasmid pVC-1 by standard calcium chloride technique (Sambrooke *et al.,* 1989) and grown in nutrient agar under ampicillin selection. Individual colonies were picked from freshly transformed

bacteria and incubated overnight at 37°C, with orbital shaking [Lab line instruments (USA), shaker bath] in Luria-Beritani medium with 0.1μl/ml ampicillin (LBamp), for DNA analysis and protein expression.

Expression of protein P32
A single colony was picked from a plate of freshly transformed bacteria and incubated overnight at 37°C with orbital shaking in 50ml of LBamp. Plasmid DNA was extracted by mini-prep method (Sambrooke *et al.,* 1989) from 1.5ml of overnight broth culture to establish colonies transformed with plasmid with insert (pVC-1). Twenty microliters (μl) of the DNA was digested with 1μl of BamHI (Life Technologies) and 1μl of EcoRI (Life Technologies) suspended in 1μl of buffer (Life Technologies) and 3μl of sterile distilled water at 37°C for 2 hours, prior to analysis on agarose gel. The growth culture with bacteria transformed with plasmid pVC-1 was diluted with 1/10 LBamp broth and incubated in 100ml aliquots in 2 liter flasks at 37°C in an orbital shaker for 3 hours. The culture was then induced with IPTG at a final concentration of 0.01mM to express the protein. The P32 protein was expressed as an intracellular protein. The cells were harvested 3 hours after the expression.

Purification of protein P32
After expression cells were harvested from the broth culture by centrifuged at 3000 rpm (Heraeus Minifuge T, West Germany, rotor size 18.4 cm) for 20 minutes at 4˚C. The supernatant was discarded and the pellet re-suspended in 10 ml of ice-cold phosphate buffered saline (PBS) per liter of medium used. The bacterial cells were lysed by rapidly freezing on dry ice followed by thawing at 37˚C. This process was repeated thrice to ensure complete lysis of the cells. The lysate was then centrifuged at 13,000 rpm for 10 minutes at 4˚C (Beckman ultracentrifuge, rotor T19) and the supernatant collected. Ten milliliters of the clarified supernatant was passed through the glutathione sepharose affinity chromatography column (Pharmacia, Biotech) at room temperature. The column was then washed three times with 20 ml of ice cold PBS and the fusion protein eluted by the addition of 10 ml elution buffer [5mM reduced glutathione (Sigma), St. Louis, (USA) in 50 mM Tris-HCl, pH 8] and collected in 1ml fractions.

Protein analysis
a) SDS-PAGE
Fifty microliters (μl) of purified protein was re-suspended in 150 μl of 2X disruption buffer [10% glycerol, 100 mM Tris-HCL, pH 7, 10% 2-mercaptothanol, 4% sodium dodecyl sulphate (SDS) and bromophenol blue] and boiled for 5 minutes. The samples were analyzed by SDS-

PAGE [Hoefer scientific instrument (USA), electrophoretic transfer unit] on a 12.5% gel using a Tris-glycine buffer system (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue and bands identified using molecular weight markers running along side the samples.

b) Western blot

The purified proteins were separated on 12% SDS – PAGE gel by use of Tris-glycine buffer system. After separation, the proteins were transferred to a nitrocellulose membrane (NCM) by electroblotting [Hoefer scientific instrument (USA), electrophoretic transfer unit]. Protein transfer was obtained within 1 hour by using 0.8 mA/sq. cm. of gel. The membrane was rinsed in PBS and immersed for 15 minutes in 5% non-fat dry milk powder in PBS to block the unsaturated sites on the NCM. The membrane was incubated with primary antibody (diluted 1/50 in 5% milk powder) at 37˚C for 1 hour and washed three times for 5 minutes in 0.05% Tween-20/PBS. The NCM was incubated with a conjugate containing horse-radish peroxidase enzyme (1/100 in 5% milk powder)(sigma) for 1 hour at 37˚C, washed as described above and transferred to 50 mM Tris-HCl (pH, 7.5) for 10 minutes. The membrane was finally incubated in freshly prepared substrate solution [10 mg of diaminobenzidin tetrahydrochloride dissolved in 50 ml of 50 mM Tris-HCl (pH, 7.5)] and 20 µl of 30 % (v/v) hydrogen peroxide for approximately 4-5 min. The reaction was stopped by washing with distilled water (Laemmli, 1970).

Western blots of the expressed protein P32 were probed with either sheep polyclonal antisera to *Capripoxvirus* [provided by Biotechnology Centre, Kenya Agricultural Research Centre (KARI), courtesy of Dr. Binepal] or with guinea pig monoclonal antisera to protein P32 (Sigma).

Inoculation of animals

Four healthy adult rabbits and six healthy adult guinea pigs of either sex were used in this study to raise antiserum to protein P32, one animal of each species served as a control. The animals were inoculated with the purified protein P32. They were injected with 250 µg of antigen per dose, intraperitoneally and by subcutaneous routes three times on day 1, 14, and 21. The antigen with complete adjuvant (Sigma) was used for the initial inoculation, and thereafter Freund's incomplete adjuvant (Sigma). The animals were then bled on day 28. The presences of antibodies against protein P32 were confirmed by VNT.

Virus neutralization test (VNT)

Virus neutralization tests were carried out using the constant serum varying virus method described by Precausta *et.al*, (1979). Serum samples were inactivated at 56˚C for 30 min, diluted 0.5 in

Glasgow minimum essential medium (GMEM) and added in duplicate columns on 96 well plates (of tissue culture grade) that had already been seeded on each well with 100µl of GMEM and 6×10^6 lamb testis cells per ml. Positive and negative reference sera were included in each plate. Virus KS-1 was added in log 10 dilutions down the plate, excluding the cell controls. The plates were incubated at 37˚C and examined daily for 9 days for CPE by light microscope.

Latex agglutination test (LAT)

The LAT's were carried out as described by Hudson and Hay (1989) with slight modifications. 100µl of the latex beads suspension were washed twice with 4 ml of 0.054mol/l glycine-saline buffer (pH 8.2) by centrifugation (Fisher scientific microfuge, Model 235V, USA) at 12,500 rpm for 10 min. After resuspension in 2.5ml of the same buffer, 100 µl of 10 mg/ml solution of the recombinant protein P32 were added and mixed for 20 min in a mixer (Fixed rotor, Hoefer model PR70). The coated latex beads were washed again twice with the same buffer and finally resuspended in 2.5 ml of 0.27 moles per liter (mol/l) glycine-saline buffer (pH 8.2) containing 0.1% of bovine serum albumin as a blocking agent and stored at 4˚C. Twenty five micro liters of two-fold dilutions of tested sera prepared in glycine-saline (0.27 mol/l) was mixed with a drop of the protein P32 coated latex beads on a clean glass slide and rocked gently while spreading the mixture for 1 min. The agglutination was read after 15 min against a white background. Clear-cut agglutination patterns with prominent blue particles against a white background were considered positive, while a uniform bluish white color was considered a negative result. The effectiveness of the sensitized LA beads was then verified using the rabbit and guinea pig antisera to protein P32.
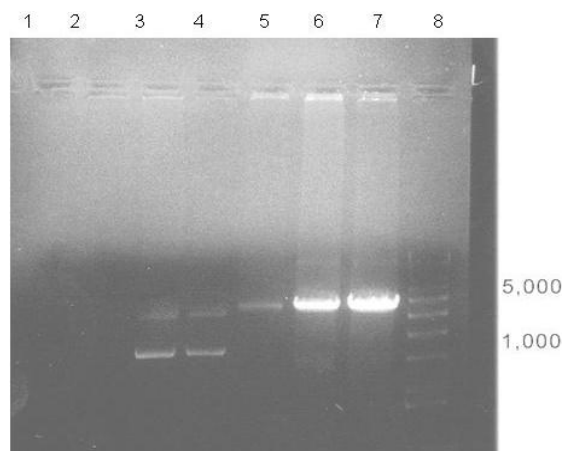

Fig. 1 Plasmid DNA analysis on agarose mini-gel following simultaneous digestion with BamHI and EcoRI. Lanes 2, 3 and 4 plasmid pVC-1 restricted with BamHI and EcoRI, Lanes 5, 6 and 7

Antiserum

One hundred serum samples of sheep, goats and cattle of unknown history were obtained from Daadab, Hiran, Middle Shabelle and Galgaduud endemic regions of Somali Republic. They were randomly picked from sera stocks held at, Biotechnology Center, National Agricultural Research Laboratories (NARL), KARI-Nairobi.

Orthopoxvirus antisera were obtained from National Veterinary Research Centre (NVRC), Muguga, KARI- Nairobi, they consisted of five pre and post inoculation sera.

Two post inoculation sera raised against *Parapoxvirus* were obtained from the Department of Veterinary Services (Nairobi), courtesy of the Director Veterinary Services.

Comparison of VNT and LAT

The rabbit and guinea pig antisera to protein (GST-P32) were used to standardize VNT and LAT. The sera samples from endemic regions, *Parapoxvirus* and *Orthopoxvirus* sera were subjected to VNT and LAT. The number of positive and negative serum samples from endemic region of Somali Republic was recorded and the data subjected to Pearson's Chi-square statistical analysis for comparison of the two tests.

RESULTS

DNA and Protein analysis

Agarose gel analysis of the restricted plasmid DNA isolated from the transformed bacteria produced two bands of 1000 and 4000 base pairs (Fig. 1). An SDS-PAGE analysis of bacterial lysate after expression produced several bands among them a

36 kDa band (Fig. 2). Two Western-blots analysis of the same lysate probed using bovine antisera to *Capripoxvirus* had three bands of 29 kDa, 32 kDa and 36 kDa (Fig. 3) while that probed with guinea pig monoclonal antisera to protein P32 had a single band of 36 kDa (Fig. 4). Analysis of the purified bacterial lysate (protein P32) by SDS-PAGE produced a single band of 36kDa (Fig. 5).

Antisera to P32 protein

The sera of rabbits and guinea pigs inoculated with the purified protein P32 were all found to have antibodies to protein P32 on the 28th day, while those of control animals did not, when analyzed by VNT (Table 1).

Development of Latex Agglutination test

Latex beads coated with the purified protein P32 when reacted with pre and post inoculation sera from rabbits and guinea pigs were found to agglutinate with the post inoculation sera but not with the pre- inoculation sera (Table 1).

Comparison of LAT and VNT

The 100 sera samples from endemic region were found to give 35 and 12 positive responses on analysis by LAT and VNT respectively. On subjecting this data to Chi-square statistical analysis at 95% level of significance and 1 degree of freedom gave a value of 14.713, P < 0.000.

Sensitivity test

The pre and post inoculation sera to *Orthopoxvirus* and the post inoculation sera to *Parapoxvirus* gave negative responses with both LAT and VNT (Table 2).

Table 1 LAT and VNT of sera from rabbit and guinea pig inoculated with P32 protein

Sample	RABBIT		GUINEA PIG	
	LAT	VNT	LAT	VNT
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	-	-	+	+
5	ND	ND	+	+
6	ND	ND	-	-

Key: + = represents positive test of either LAT or VNT, - = represents negative test of either LAT or VNT, ND=represents not done

Table 2 Latex agglutination test and virus neutralization test on antisera to *Parapoxvirus* and *Orthopoxvirus*

	Sample	ORTHOPOXVIRUS		Sample	PARAPOXVIRUS	
		LAT	VNT		LAT	VNT
PRE	2	-	-	1	ND	ND
	5	-	-	2	ND	ND
	6	-	-			
	7	-	-			
	14	-	-			
POST	2	-	-	1	-	-
	5	-	-	2	-	-
	6	-	-			
	7	-	-			
	14	-	-			

+ = represents positive test of either LAT or VNT, − = represents negative test of either LAT or VNT, ND = represents not done
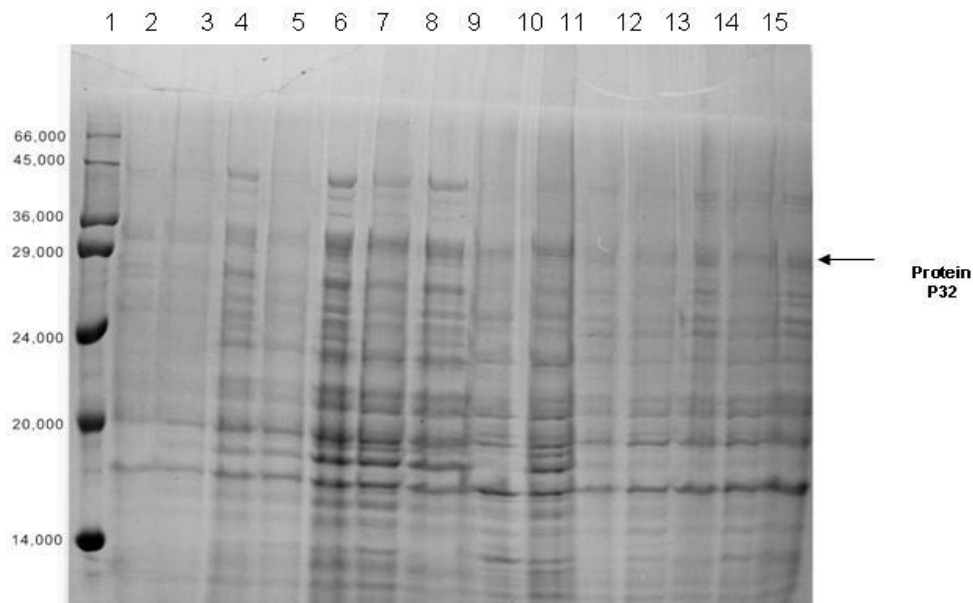
Fig. 2. **SDS-PAGE analysis of bacterial lysate following IPTG induced expression of pVC-I transformed E.*coli*.** Lane 1, Protein molecular weight marker; Lanes   2 and 3 zero hour protein expression sample, Lanes 4, 5, 10 and 11 one-hour protein expression samples; Lanes 6, 7, 12 and 13 two-hour protein expression samples; Lanes 8, 9, 14 and 15 three-hour protein expression samples; Arrow shows the position of the fusion protein (Glutathione-P32) **(Molecular wgt. range 14,000-70,000)**

Fig. 3. Western blot analysis of bacterial lysate following IPTG induced expression of pVC-I transformed E.*coli* using sheep polyclonal antisera to *Capripoxvirus*. Lanes   2 and 3. Zero hour protein expression sample; Lanes 4 and 5 one-hour protein expression sample; Lane 10 (M) protein molecular weight marker; Lanes   6, 7, 11 and 12 two-hour protein expression sample; Lanes 8, 9, 13, 14 and 15 three-hour protein expression sample; Arrow shows the position of the fusion protein (GST-P32).The protein molecular weight marker used in lane 10 was   obtained   Sigma   Dalton   mark   VL   (Molecular   wgt.   range   14,000-70,000)
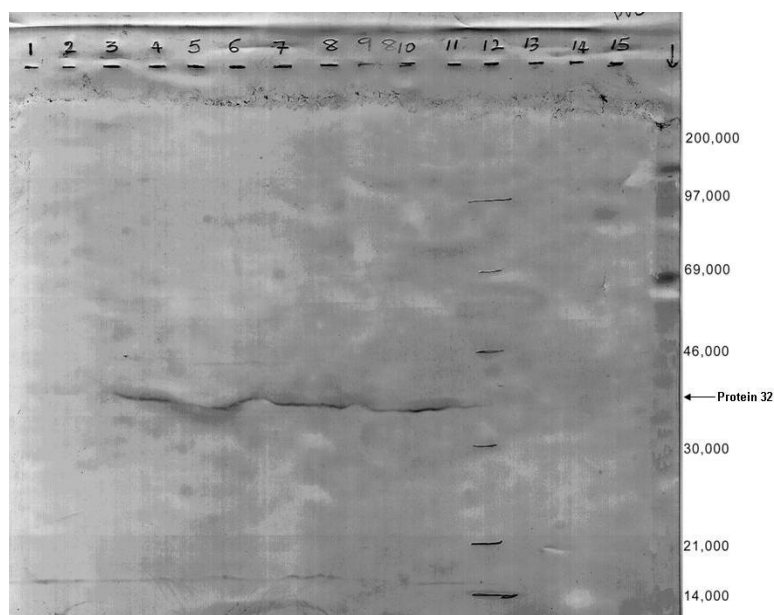
Fig 4. Western blot analysis of bacterial lysate following IPTG induced expression of pVC-I transformed E.*coli* using guinea pig monospecific antisera to protein P32. Lane 2 = zero hour protein expression sample, Lane 4 = one-hour protein expression sample, Lane 6 = two-hour protein expression sample, Lane 8 = three-hour protein expression sample, Lane 10=four-hour protein expression sample, Lane 12=protein molecular weight marker, Lane 15 = E. *coli* without plasmid, Arrow ⟶ shows the position of the fusion protein (GST-P32). The protein molecular weight marker used in lane 12 was obtained from Sigma High molecular weight standard mixture (Molecular weight 30,000-200,000)
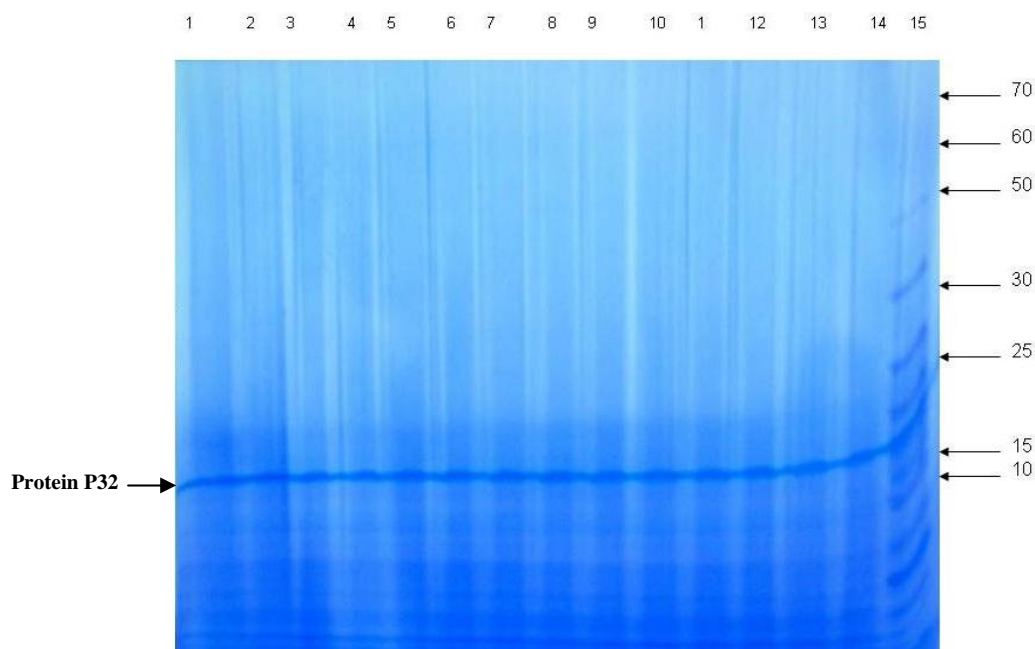

Fig. 5 SDS-PAGE analysis of eluate from bacterial lysate following IPTG induced expression of pVC-I transformed E.*coli* passed through glutathione sepharose affinity chromatography column eluted with 5mM reduced glutathione in 50mM TRIS - HCl pH 8.

DISCUSSION

The previous serological tests were slow and tissue culture based, this caused the tests to have different viral titres and therefore could not be used as reference tests (Kitching, 1992). Also an ELISA developed gave false positive and high positive (Ngichabe, 2002). There was therefore a need to improve on the tests. The isolation of plasmid DNA from transformed *E.coli* and its subsequent digestion with restriction enzymes revealed two bands of 1000 and 4000 base pairs corresponding to those of gene Q_13_L and plasmid PGEX-2T respectively (Fig. 1). This indicated that the bacteria were effectively transformed with plasmid pVC-1, confirming earlier work by Chand P. (1992).

The recombinant fusion protein glutathione-s-transferase-P32 (GST-P32) was produced as an intracellular protein, therefore, after expression the bacteria were lysed to release the protein. Analysis of the bacterial lysate by SDS-PAGE produced several bands among them a 36 KDa band corresponding to that of the protein GST-P32. Further analysis of the lysate by Western-blot probed with sheep post inoculation sera to *Capripoxvirus* produced three bands of 29, 32 and 36 KDa. A similar blot probed with guineapig monoclonal antisera to P32 produced a single 36 KDa band. This indicated that fusion protein GST-P32 was successfully expressed and that the 29 and 32 kDa bands were bacterial proteins (Fig. 4). Therefore use of the lysate (unpurified P32) to sensitize latex agglutination beads would give false positive and high positive result as observed by Ngichabe *et. al.* (2002) while using the lysate to developed an immunocapture ELISA to test for CPV antibodies. It was not possible to cleave the fusion protein, the difficulty encountered is not unusual as it has been experienced by others using pGEX-2T as an expression vector (Dr. Y. Binepal, personal communication). GST does not raise significant levels of antibodies that would interfere with the test when used as a fusion protein with P32 (Carn, 1994).

Purification of lysate by affinity chromatography column and its subsequent analysis using SDS-PAGE produced a single band of 36 KDa, indicating that the purification of the fusion protein P32 was successful (Fig. 5), this is in conformity with earlier work by Carn V. (1994). This was further confirmed by non agglutination of pre inoculation sera and agglutination post inoculation sera from guinea pig and rabbit respectively (Table 1). It also indicated that the sensitized beads were sensitive to and effective in detection of antibodies raised against the purified protein P32.

Subjection of the 100 sera samples from endemic region to VNT and LAT and subsequent comparison of the data obtained with Pearson's Chi-square statistical analysis showed that the two tests were significantly different at one degree of freedom and 0.05 level of significance. This is because the calculated Chi-square value of 14.7 was greater than that of the tabulated value of 3.841, signifying that the LA test is 23% more sensitive than VNT.

Orthopoxvirus and *Parapoxvirus* antisera cannot be distinguished from CPV antisera by some serological tests (Davies, 1982; Kitching, 1986), but the developed LAT is able to distinguish them as demonstrated by failure of the post-inoculation sera of *Orthopoxvirus* and *Parapoxvirus* to agglutinate with the sensitized LA beads (Table 2). This indicates that the LAT is specific for *Capripoxvirus* antibodies. This is in agreement with earlier work by Rao *et. al.* (1997) using whole viral particle to coat LA beads found the test to be specific for CPV antibodies.

The LA test provides a rapid, sensitive and specific diagnostic test for CPV infection as compared to other serological tests. Its reliability, affordability and ease of use in areas with minimum facilities makes it a diagnostic test of choice particularly in places that are remote or without highly qualified personnel. The test can also be used in markets within the country and export markets to verify the condition of the animals.

REFERENCES

Carn V. M. 1993. Control of capripoxvirus infections. Vaccine, 11 (13) 1275-1279; 56 ref.Carn V. M. (1994). The experimental transmission and diagnosis of lumpy skin disease (Neethling) PhD. Thesis. University of Reading, England.

Carn, V.M. (1995). Antigen trapping ELISA for the detection of *Capripoxvirus* in tissue culture supernatant and biopsy samples. Journal of Virological methods, 51: 95-102.

Chand P. (1992). Molecular and immunological characterization of a major envelop protein of Carpripoxvirus. PhD. Thesis. Research unit, Department of Agriculture, University of Surrey.

Davies, F. G. (1976). Characteristics of a virus causing a pox disease in sheep and goats in Kenya, with observations on the epidemiology and control. *Journal of Hygiene, Cambridge,* **76**: 163-171.

Davies, F. G. (1982). Observations on the epidemiology of lumpy skin disease in Kenya. *Journal of Hygiene, Cambridge* **88**: 95-102.

Heine H. G., Stevens M. P., Foord A. J. and Boyles D. B. A Capripoxvirus detection PCR and antibody ELISA

based on the major antigen P32, the homolog of the vaccinia virus H3L gene. J Immunol Methods. (1999) Jul 30; **227**(1-2): 187-96.

Hosamani M., Mondal B., Tembhurne P. A., Banddyopadhyay S. K.,Shingh R. K. and Rasool T. J. Virus Genes. 2004 Aug; 29 (1): 73-80

Hudson, L. and Hay, F. C. (1989). Practical Immunology. *3ʳᵈ Edition, Blackwell Scientific Publication*, Oxford- London- Edinburg- Melabourne, pp 257-258.

Kitching R. P. Vaccine for lumpy skin disease, sheep pox and goat pox. Dev Bio (Basel) 2003; **114**: 161-7

Kitching, R. P. and Hammond, J. M. (1992). Poxvirus, infection and immunity. *Encyclopedia of immunology*, vol.**3**: 1261-1264 Ed. Roith, I. M. and Delucs, P.J. London Academic Press.

Kitching, R. P., Hammond, J. M. and Black, D. N. (1986a). Studies on the major common precipitating antigen of Capripoxvirus. *Journal of General Virology*, **67**: 139-148

Kitching, R. P. and Smale, C. (1986b). Comparison of the external dimensions of *Capripoxviruses* isolates. *Research of Veterinary Sci*ence, **41**: 425-427.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head bacterialphage T4. Nature (London) **227**:680-685

Ngichabe, C.K., Binepal, Y.S., Njiru, J.W. and Carn, V.M. (2002). Evaluation of an enzyme linked Immunosorbent assay for detection of Capripoxviruses. *Short communication*, Kenya Agricultural Research Institute.

OIE (Office international des Epizooties) Manual of Diagnostic Tests and vaccine for Terrestrial Animals. Chapter 2.1.10 (2004).

OIE (Office international des Epizooties) Manual of Diagnostic Tests and vaccine for Terrestrial Animals. Chapter 2.1.10 (2005).

OIE (Office international des Epizooties/ World of Animal Health). Sheep pox and goat pox. In: Technical disease card database 2002.

Plowright, W. and Ferris, R. D. (1958). The growth and cytopathogenicity of sheep-pox virus in the tissue cultures. Britain Journal of Experimental Pathology, **39**: 424-435.

Precausta, Kato F. and Vellut, G. (1979). A new freeze-dried living virus vaccine   against sheep-pox. Compendium of Immunology, Microbiology and infectious Diseases **1** 309-319.

Rao V. T., Malik P., Nandi S. and Negi B. S. Evaluation of immunocapture ELISA for diagnosis of goat pox. Acta Virol (1997) Dec; **41**(6): 345-8

Rao, T. V. and Bandyopadhyay, S. K. A comparison review of Sheep pox and goat pox and their diagnosis. Anim Health Res Rev. 2000 Dec; **1**(2): 127-36.

Sambrook, J., Fritsch, E. F and Maniatis, T. (1989). Molecular cloning: A laboratory manual. 2ⁿᵈ Edn. Cold Spring harbor Laboratory Press.

Sharma B., Ngei B. S., Pandey A. B., Banddyopadhyay S. K., shankar H., Yadav M. P. (1988). Detection of goat pox antigen and antibody by counter immunoelectrophoresis test. Tropical animal Health and Production, 20(2): 109-113; 8 ref.

Srivastava, R. N. and Singh, I.P. (1980). A study on the role of cellular and humoral factors in immunity to sheeppox. *Indian Journal of Animal Science,* **50**: 861-866.

Subba Rao M. V., Malik, B. S. and Sharma, S. N. (1984). Antigenic relationships among sheeppox, goatpox, and contagious pustular dermatitis viruses. *Acta Virologica,* **28**: 380-387.

Van Regenmortel H. V., Bishop D. H. L., Van Regenmortel M. H., Claude E. Fauquet (Eds). VirusTaxonomy: eighth report of the international committee on taxonomy of viruses (2005).

Lead (Pb) uptake (mediated by Arbuscular Mycorrhiza) from Soil and Pb Deposit on Cowpea (*Vigna unguiculata* [L.] Walp) Plant

Taiwo, L. B.

Soil Microbiology Unit, Institute of Agricultural Research and Training, Obafemi Awolowo University, P. M. B. 5029, Ibadan., E-mail: lbtaiwo@yahoo.com

Abstract
The need to increase cowpea production for the increasing population in cities had led to cultivation of available land in city centres for farming regardless of the level of heavy metal pollution of such land. It has therefore become imperative to assess this practice with the aim of identifying the level of danger inherent in the practice. A glasshouse experiment was conducted to assess the level of tolerance of cowpea (*Vigna unguiculata), Ife Brown* variety to lead (Pb). The seeds were inoculated with *Glomus mosseae* (Nicolson & Gerdemann) to enhance nutrient and Pb uptake before planting in soil enriched with lead (Pb). A field study to determine the response of same crop to Pb fallout from automobiles was also carried out. The field experiment was sited beside a major road leading to a petroleum deport in Ibadan, Nigeria. Vegetative growth of the crop was improved by the microorganism. Though Pb impacted negatively on cowpea growth, only the application of the combination of Pb and G. *mosseae* led to stunted growth of the crop. In the field, soil Pb decreased as we moved away from the road embankment with soil 15 m away from the road-side containing 17.3 mgkg^{-1} while plot closest to the road had 82.3mgkg^{-1}. Strong correlations existed between soil Pb and root Pb (r=0.918), soil Pb and plant tissue Pb (r=0.973) and between root Pb and plant tissue Pb (r=0.913). The Pb content of root of cowpea at 4, 6, 8,10 and 15 m away from the road-side were lower than the corresponding values in plant tissues indicating that cowpea tissue might have obtained its additional Pb from other sources than from the soil. Cowpea showed some level of tolerance to Pb as only the combination of Pb and G. *mosseae* led to visible stunting in growth.

Keywords: Cowpea (*Vigna unguiculata*); *Glomus mosseae*; rhizobium; lead

Introduction

In the urban and peri-urban environments of developing countries, cropping of marginal city soils for food production has been on the increase. Retired civil servants and resource-poor city farming community cultivate spent soils to produce crops to meet domestic food demand. Constrained by stiff competition for limited land, city farmers have now shifted to available roadside land spaces to cultivate vegetables, maize and cowpea, disregarded the risks involved in such practice. One of the risks involved is the heavy metal deposits on crop plants by vehicular exhausts. In recent times, there had been considerable interest in heavy metal accumulation in food crops especially those grown on the roadsides. Lead (Pb) is one of the most important of these metals. The danger to health from Pb residue in soil and dust is now widely accepted. According to Arrouays et al. (1995), the danger of Pb derived from vehicle and dust to health is getting to an alarming proportion. Some of the long-lasting adverse neurobehavioral effects are irreversible even when symptoms of Pb poisoning are clinically corrected (Farfel, 1985; Page and Chang, 1993). Many other anthropogenic activities have already resulted in widespread Pb contamination of surface soils (Haygarth and Jones, 1992). Heavy emitters of airborne particles have been identified as a source of severe pollution of agricultural and urban soils. In the process of metal deposits on soils, plant parts receive a high proportion of the metals thereby increasing their accumulation. The consequence is the continuous accumulation of these metals and their persistence in the food chain. One technique with potential of reclaiming some of toxic wastes is to make use of heavy metal tolerant plants (Wu, 1989)

One crop that has been found to be tolerant of Pb is cowpea (*Vigna unguiculata* (L) Walp) (Sudhakar *et al*. 1992). Apart from its ability to tolerate draught conditions and its capacity to obtain nitrogen (N) nutrition through the biological nitrogen fixation, it also has the potential to mobilize soil nutrients through symbiotic partnership with arbuscular mycorrhiza (Taiwo and Oladapo, 1999; Taiwo *et al*.2000). It has therefore, become necessary to

examine the ability and potential of cowpea to take up Pb from soil.

Materials and Method

The study was carried out in two phases: 1.) Glasshouse conditions to assess the level of tolerance of aided and un-aided growing cowpea when grown in a Pb-enriched soil and 2.) Field conditions where the experiment was located on the roadside of a major road leading to a petroleum depot. The objective of the field study was to distinguish between Pb deposit on plant part and Pb uptake by the plant root.

Glasshouse Experiment

The experiment was comprised of three factors. The main factor was the soil status, either sterilized or un-sterilized. The main objective of sterilizing the soil was to reduce the presence of nematodes and some other disease agents in the soil. In the process of heat treatment, some useful microorganisms in the soil involved in the nutrient mineralization were expected to be eliminated and so reduce the availability of such nutrients. The impact of this may be profound and therefore required investigation hence listed as a factor. The second factor, mychorriza inoculation consisted of *Glomus mosseae* (Nicolson & Gerdemann), or a non-inoculated control. Results from earlier experiments (Taiwo and Oladapo, 1998 and Taiwo *et al.* 2000) indicated that *Glomus mosseae* was effective in nutrient uptake. The third factor was presence or absence of Pb in soil.

Treatment Applications

Mychorrizal Inoculation

Fifty (50) grams of the soil culture of G. *mosseae* which consisted of mycelia, hyphae and spores were used to inoculate the seeds of cowpea. The inoculum was obtained from Botany and Microbiology Department, University of Ibadan, Nigeria. The fifty gramme crude inoculum of the mycorrhiza; obtained from soil in which maize was used as trap crop was introduced into the planting holes of the inoculated pots while the same quantity of attenuated inoculum was applied into the planting hole of non-inoculated pots. All the treatments received peat-based rhizobium inoculum IRc 284 collected from the International Institute of Tropical Agriculture (IITA). Four seeds of the cowpea coated with the inoculant were planted. The seedlings were thinned to 2 stands per pot, 2 weeks after planting (WAP). Percentage root infection was

determined according to the grid line intersect technique of Goivanetti and Mosse (1980).

Table 1. Pre-cropping soil Analysis in the glasshouse experiment

Parameters	Values
C (g kg^{-1})	8.3
N (g kg^{-1})	0.7
Available P (mg kg^{-1})	3.41
Mechanical Analysis	
Sand (g kg^{-1})	840
Silt (g kg^{-1})	100
Clay (g kg^{-1})	60
Exchangeable Bases (C mol kg^{-1})	
Ca	2.4
Mg	1.6
K	0.19
Na	0.63
CEC	4.95
H+	0.13
pH	5.5
Base saturation (%)	97
Pb (mg kg^{-1})	8

Lead (Pb) Application

Lead was applied as 0.366 g^1. The soil used in the glasshouse study was, enriched with Pb to provide adequate quantity of the metal to achieve the objective of the study. Lead Ammonium Acetate (PbNH$_4$C00$_3$) was added into each 5 kg soil to make up to 200 mgkg^{-1} of Pb content. High concentration of Pb was introduced to test and confirm the tolerance of cowpea to Pb. The plants were allowed to grow for a period of 10 weeks before harvest. At 6 WAP, nodule number and weight were recorded. Similarly, the plant vegetative parts (shoot and root) were harvested, dried and ground. Plant nitrogen (N) was determined by Kjedahl digestion method (Eastin, 1978), phosphorus (P) was by Chloromolybdate blue method and potassium (K) content was read on flame photometer. Lead (Pb) content of the root and shoot were also determined using Atomic Absorption Spectrometer (AAS). At maturity, (10 WAP) the seed yield parameters were recorded. At harvest (10 WAP), seed yield were determined.

Field Experiment

The field experiment, replicated in three sites, examined the fallout of Pb on road-side soils along a major road leading to a petroleum product depot of Nigerian National Petroleum Corporation (NNPC) in Ibadan, Oyo State, Nigeria.

Table 2: Pre-cropping soil analysis of road-side soil

Parameters	Values
N (g kg^{-1})	2.8
Available P(mg kg^{-1})	11.65
C (g kg^{-1})	24.5
Exchangeable cations(C mol kg^{-1})	
Ca	.61
Mg	.42
Na	.28
K	.64
H+	.13
pH	6.9
CEC (C mol kg^{-1})	5.31
Base saturation (%)	97
Mechanical Analysis	
Sand (g kg^{-1})	800
Silt (g kg^{-1})	140
Clay (g kg^{-1})	60
Micronutrients (mg kg^{-1})	
Fe	1.34
Zn	7.40
Cu	20.20
Heavy metals (mg kg^{-1})	
Mn	690
Cd	0.10
Cr	40.2

The three sites, 10 metres apart along the NNPC road were selected for the experiment. The sites were closely selected to minimize variability in the soil characteristics. At each site, five plots, consisting of 2 by 2 m each at 2, 4, 6, 8,10 and 15 m away from the road embankment were marked out. In between the plots were inter-plot spaces of 1 m. The objective in varying the distances from the roadside was to examine distribution of lead as distance of plots increased from the embankment. Pre-cropping soil samples from each of the sites and plots were taken, analyzed and the results presented in table 1. The soils from plots in each of the sites were analyzed separately and results of the sites and plots were subjected to statistical analysis using COSTAT package. The Pb contents of each of the sites and plots were statistically analyzed and results subjected to regression analysis to determine the effect of distance on the level of Pb.

Cowpea (*Vigna unguiculata*), Ife Brown was planted after land preparation. Inter-row spacing was 60 by 60 cm, while the space within the row was 30 cm. At 8 weeks after planting, plant samples were taken, dried at 70°C, ground and digested to determine the Pb composition using the atomic absorption spectrometer (AAS). The results are presented in table 2.

Results
Lead content (8 mg kg^{-1}) was relatively low in the soil used in the glasshouse experiment. Heat treatment of soil as done in the glasshouse led to reduction in vegetative and seed yield parameters (Fig. 1). From figure 2, *G. mosseae* increased vegetative as well as nodule weights of cowpea relative to control. Fig. 2 also shows that enrichment of soil with Pb decreased nodule and vegetative weight of plant relative to *G. mosseae* inoculated plant. Lead alone significantly depressed the weights of root and seed relative to *G. mosseae*-inoculated plant as shown in Fig. 3. Similarly, the interaction effect of Pb and *G. mosseae* significantly reduced the weights of root and seeds relative to the effect of *G. mosseae* and control. Fig. 3 also show that *G. mosseae* inoculation led to significant increase in weights of root and seed relative to control.

Weight of nodules of cowpea inoculated with G. *mosseae*+ Pb was smaller than the weight of the nodules when the plant was growing under the influence of the G. *mosseae* alone. Further studies are required to ascertain the mechanisms of action of this interaction. Though Pb reduced growth and yield of cowpea in the glasshouse, only the application of the combination of G. *mosseae* and Pb led to stunted growth of the crop. Tissue contents of nitrogen (N) and phosphorus (P) varied with the treatments (Table 3).

Glomus mosseae-inoculated cowpea plant tissue contained the highest level of N. Lead+*G. mosseae*-treated cowpea plant tissue contained the highest level of P. Lead was not detected in the shoot of cowpea even though the soil supporting such cowpea was treated with lead acetate. Only the roots of the crop contained Pb in spite of the fact that the roots were properly washed in sterilized and distilled water.

Table 3: Nitrogen (N), Phosphorus (P) in Plant shoot and Pb contents in vegetative shoot and root of cowpea plants harvested in the glasshouse experiment

Treatments	Shoot		Shoot	Root	Root infection (%)
	%N	%P	Pb (mg kg^{-1})	Pb (mg kg^{-1})	
Control in sterilized soil	2.47^c	0.03^a	ND	ND	10^a
Glomus mosseae in sterilized soil	2.71^{ef}	0.04^a	ND	ND	67^b
Control in un-sterilized soil	2.53^{cef}	0.06^b	ND	ND	12a
Glomus mosseae in un-sterilized soil	2.40^{cd}	0.09^c	ND	ND	65^b
Pb in sterilized soil	2.32^{bc}	0.06^b	ND	85.0^a	8^a
Pb+*Glomus mosseae* in sterilized soil	2.57^{cef}	0.10^{cd}	ND	97.0^b	68^b
Pb in un-sterilized soil	2.22^{bd}	0.07^b	ND	102.0^b	9^a
Pb+*Glomus mosseae* in un-sterilized soil	2.00^a	0.11^d	ND	40.0^a	69^b
CV%	20.45	10.25	-	15.20	19.2

ND = **Not Detectable**

It is assumed that the adverse effect exhibited by Pb was restricted to the root region. It is even possible that the presence of Pb did interfere with N but not with P uptake. As for root infection with *G. mosseae* (Table 3), un-inoculated plants had very low percentage infection. *G. mosseae*-inoculated plants had percent infections that were more than 80% higher than un-inoculated plants. *G. mosseae*-inoculated cowpea in un-sterilized soil had the highest infection percentage.

In the field experiment (Table 4), the soil, cowpea root and shoot-Pb decreased as we moved further away from the road embankment. Soil consistently contained the highest level of Pb in all the plots as against the values in the root and shoot. Soil Pb was over 4 times the value of Pb in cowpea root and over 5 times the value of Pb in cowpea shoot in the same plot. There were strong correlations between soil Pb and root Pb (r =0.918), between soil Pb and shoot Pb (r =0.973). Though, the correlation between root Pb and plant shoot Pb (r =0.913) was also strong, the Pb content of root of cowpea at 4, 6, 8,10 and 15 metres away from the embankment (4.0 1.7, 0.6, 0.3 and 0.1 mgkg^{-1}) were lower than the corresponding values in plant shoot (5.0, 2.9, 1.7, 0.5 and 0.2 mgkg^{-1}).

Table 4: Pre-cropping Pb content in soil, root and plant tissue (Field experiment)

Distance from road embankment (m)	Soil Pb (mg kg^{-1})	Root Pb (mg kg^{-1})	Plant shoot (mg kg^{-1})
2.0	82.3^a	7.5^a	7.0^a
4.0	68.6^b	4.0^b	5.0^b
6.0	53.3^c	1.7^c	2.9c
8.0	34.1^d	0.6^d	1.7^d
10.0	22.0^e	0.3^{de}	0.5^e
15.0	17.3^e	0.1^e	0.2^e
CV (%)	10.0	23.0	13.01

Discussion

Results of soil chemical analysis prior to planting of cowpea in table 1 showed that the nutrients are low especially nitrogen (N) and phosphorus (P) for cowpea growth. Soils containing 1.0 and 1.5 g.kg^{-1} N and 14mg.kg^{-1} P is considered to be of minimum fertility status (Sobulo and Adepetu, 1987). The reduction in some parameters in plants grown in heat treated soil in the greenhouse was expected. This might be a consequence of adverse effect of heat on both intra and extra-cellular microbial enzymes of the killed microorganisms. Other reason which might be adduced is the negative impact of heat on the Mycorrhiza Helper Bacteria (MHB) as well as reduction in the number of nutrient mineralizing organisms.
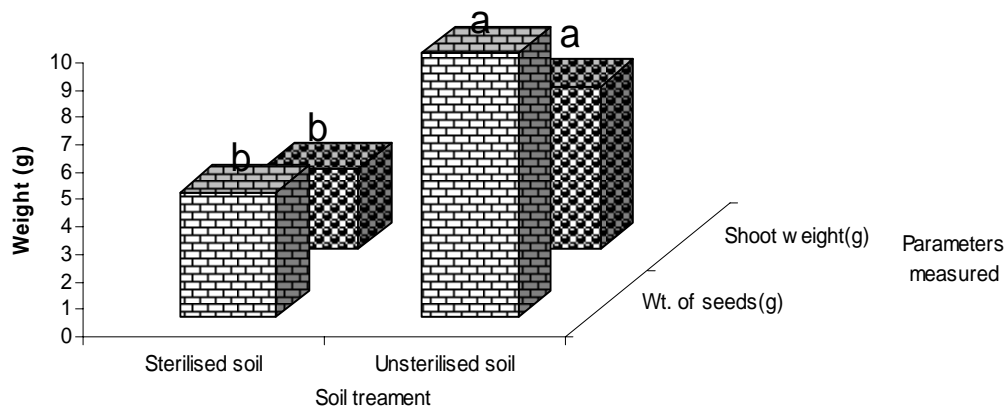
Fig. 1: Influence of soil treatment on plant vegetative and seed yield.
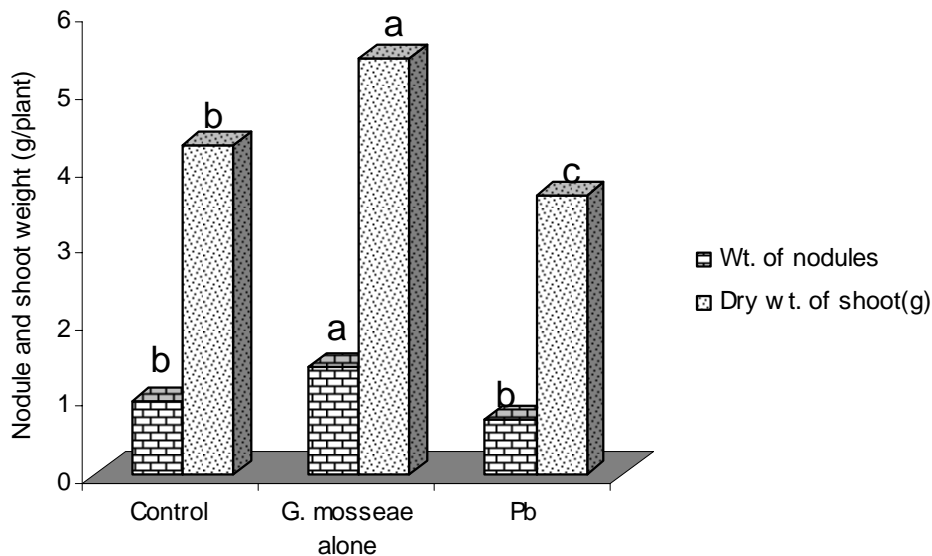
Fig. 2: Effect of Pb and *G. mosseae* on weight of nodule and shoot (g/plant)

In this study, *G.* mosseae enhanced nodule, root, shoot and seed weights of cowpea. It had been observed that mycorrhiza infection enhances plant growth by increasing nutrient uptake via increases in the absorbing surface area. According to Tinker (1984), mycorrhiza increases uptake of nutrients such as potassium, sulphate, copper and zinc in addition to phosphorus.

Although *G. mosseae* usually enhance uptake of P and some other nutrients in soil, the combination of *G. mosseae* and Pb might have impaired cowpea growth. Apart from the adverse effect of Pb, some of the photosynthates of cowpea might have been lost to *G. mosseae*. According to Jacobson *et al.* (1990), for all the beneficial activities that plant derive from mycorrhiza, it has to pay a price of between 10 and 20% of the net photosynthates that are required for the formation, maintenance and function of mycorrhizal structures.

of Pb in cowpea plant tissue top indicates very minimal presence of these forms in the soil used.

No significant impact of Pb or the distance from the road embankment was observed on the vegetative and grain yields. This can be attributed to possible dilution of the metal as the upward transport progressed. Results in this study showed that the effect of Pb was mainly shown in cowpea root both in the glasshouse and in the field indicating that cowpea shoot possesses some level of tolerance to Pb. The results above however, provide sufficient evidence to conclude that mycorrhizal inoculant enhances Pb absorption by or adsorption on the roots in soils enriched with Pb. However, Pb fallout on plant parts from the atmosphere especially from passing automobiles may constitute a ready source of this metal in plant top tissue. Further studies are required to assess the mechanism of exclusion of Pb when cowpea take-up other nutrients.
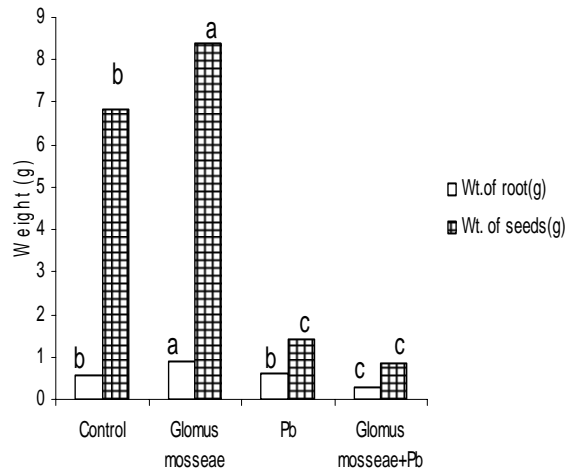

Fig. 3: Effect of Pb and *G. mosseae* interaction on weight of root and seed (g/plant)

In the field study, it was shown that soil contained more of heavy metals than the root and shoot of cowpea plant. Alloyway, (1990) showed that lead was strongly retained in surface horizons of the soil profile because of its adsorption by clay mineral, manganese (Mn) and iron (Fe) oxides and organic matter. Lead content in the deeper layer is therefore, probably due to both geochemical content and diffuse pollution, whereas Pb content in the upper soil layer (0-0.2m depth) may mostly be due to local fallout (Arrouays *et al*., 1995).

Increases in Pb content in plant tissue top as against the lower content of root Pb provides a sufficient evidence to assume that cowpea tissue may have obtained its additional Pb from the atmosphere- presumably from fumes from vehicular exhaust pipes. Sudhaker *et al*. (1992) reported Pb tolerance of certain legume species grown on Pb ore tailing and noted that cowpea showed a higher Pb-tolerance index than other legumes. Lead or any other heavy metal uptake is highly influenced by the forms such metals exist in the soil. The total content is often of little importance in calculating the quantity which can be taken up by plants and consequently cause phytotoxicity or enter the food chain, whereas the chemical form of a heavy metal in the soil is of particular interest. The simple or complex ions, the exchangeable and the organic forms are considered to be the most available forms for plant nutrition (Petruzzelli, 1989). The zero level

References
Alexander, M. (1977). Some Aspects of Microbial Physiology. In: Introduction to Soil Microbiology. John Wiley & sons, Inc. New York p115-127.

Alloway, B. J. (1990). Heavy metal in Soils. John Wiley and Sons, New York. p339.

Arrouays, D.; Mench, M; Annas, V and Gomez, A. (1995). Short Range Variability of Fallout Pb in a Contaminated Soil. *Canadian Journal Soil Science*. 76:73-81.

Eastin, E. R. (1978), "Total nitrogen determination for plant material", *Annal of Biochemistry*, 85:591-594

Farfel, M. R. (1985). Reducing lead (Pb) Exposure in Children. *Annual Review of Public Health 6: 333-360.*

Giovanetti, M. and Mosse, B. (1980). An evaluation of technique for measuring vesicular arbuscular mycorrhizal infection in roots. *New phytopathology*. 84:489-500.

Haygarth, P. M. and Jones, K.C. (1992). Atmospheric Deposition of Metals to Agricultural Surfaces. Pages 249-276. *Biochemistry of Trace Metals* (ed. D.C.Adriano).In: Biogeochemistry of trace metals. Lewis Publishers, Boca Raton , FL.

Jacobson, I and Rosendahl, L. (1990). Carbon flow into soil and external hyphae from root of mycorrhiza Cucumber plants. *New Phytopathology Journal* 115: 77-83

Page, A.L. and Chang, A.C. (1993). Lead Contaminated Soils: priorities for remediation. Hazardous Waste and Hazardous Materials 10: 1-2.

Petruzzelli, G. (1989). Recycling Wastes in Agriculture: Heavy Metal Bioavailability. Agriculture, Ecosystem and Environment 27: 493-503

Sobulo, R. A. and J. A. Adepetu. 1987. Soil testing and fertilizer formulation for crop production in Nigeria. National Fertilizer Seminar, Port Harcourt, 28-30 October 1987.

Sudhakar, C; Syamalabai, L and Veeranjaneyulu, K. (1992). Lead Tolerance of Certain legumes species on Lead Ore Tailings. *Agriculture Ecosystem and Environment. 41: 253-261.*

Taiwo, L. B. and Oladapo, M. O. (1999). Response of Cowpea (*Vigna unguiculata*) to AM and *Bradyrhizobium* in a Heavy and Light-Textured Soil. *Tropical Agricultural Research and Extension. 2:91-94*

Taiwo, L. B.; Osonubi, O. and Salau, M. (2000). Growth and Nodulation of Cowpea (*Vigna unguiculata*) to Compost Application and Inoculation to *Glomus mosseae* and *Bradyrhizobium. Journal of Agriculture. and Environment for International Devpt*. 94: 21-34.

Taiwo, L. B.; Osonubi, O and Akano, M. J. (2001). Growth and Nodulation of Cowpea *(Vigna unguiculata*) in Response to Compost and Inoculation with Glomus etunicatum and Bradyrhizobium. *Tropical Agricultural Research and Extension*. 4: 101-107.

Tinker, P. B. (1984). Endomycorrhizas. *Australian.Journal of Plant and Soil*. 76:77-80

Wu, L. (1989). Colonization and Establishment of Plants in Contaminated Sites. In: A.J. Shaw (Editor), heavy Metal Tolerance in Plants. CRC Press, Boca Raton, FL,pp. 269-284.

Evaluation of *Verticillium chlamydosporium* and *Arthrobotrys oligospora* for biological control of *Meloidogyne incognita* in celery and tomato

[1]Nyongesa, M.W., [2]J. Coosemans and [3]J. W. Kimenju.
[1]Corresponding Author: National Potato Research Centre, KARI-Tigoni. P.O. Box 338 Limuru. E-mail:
mnyongesa@hotmail.com
[2]Katholieke Universiteit, Leuven, Laboratory for Phytopathology and Plant Protection, W. De Croylan 42, B-3001 Hervelee,
BELGIUM
[3]University of Nairobi, Crop protection Department, P.O. Box 29053 Nairobi, KENYA

Abstract

The ability of nematode trapping fungi and egg-parasitising fungi to colonize and persist in the rhizosphere of crop plants is thought to be an important factor influencing the success of biological control of root infecting nematodes. In this study, two strains of an egg parasitic fungus *Verticillium chlamydosporium* (Vc-10 and Vc-2M) and one isolate of the nematode-trapping fungus *Arthrobotrys oligospora* were evaluated to determine their pathogenicity to *Meloidogyne incognita* and persistence in the rhizosphere of celery (*Apium graveolus* L.) and tomato(*Lycopersicon esculentum* L.) plants in a greenhouse experiment. The isolates tested differed significantly (P≤0.05) in their pathogenicity to *M. incognita* and survival in the rhizosphere. Isolate Vc-10 of *V. chlamydosporium* was the most virulent pathogen of the nematode. Root galling was lowest (P≤0.05) in tomato plants treated with the isolate Vc-10 (2.2); pots treated with this isolate had the lowest final soil population of infective juveniles; there was a 62.2% and 98.5% infections of eggs and egg-masses respectively by Vc-10 on tomato plants. Isolates Vc-10 and Vc-2M of *V. chlamydosporium* persisted in the soil and could be re-isolated from the rhizosphere and roots of tomato plants at least 16 weeks after soil infestation. The final inoculum density was, however, higher (P≤0.05) for Vc-10 (1.35×10^5 cfu/g soil) than Vc-2M (9.25×10^4 cfu/g soil). *A. oligospora* on the other hand did not give any significant (P≤0.05) control of the nematode on both crops, there was severe galling on the roots of celery plants (7.4) and tomato plants (6.3) treated with this biological control agent. *A. oligospora* could not be re-isolated from the plant rhizosphere sixteen weeks after soil infestation. Lack of nematode control on both crops by *A. oligospora* was attributed to its poor or no establishment in the plant rhizosphere. The fact that it could not be re-isolated from the rhizosphere may imply that the fungus did not survive in the rhizosphere of the test plants.

Key Words: *Verticillium*, *Arthrobotrys*, *Meloidogyne*, rhizosphere, persistence, root-knots, chlamydospores.

Introduction

Plant parasitic nematodes especially root-knot nematodes (*Meloidogyne* spp.) are an important group of pests that cause severe damage to crops especially in tropical subsistence agriculture (Bridge, 1987). Root-knot nematodes cause galling on the roots of infected crops leading to poor growth and reduced yield. A world survey with 371 responses from nematologists resulted in an estimation of annual loss for the 21 investigated crop species to be about $77 billion. *Meloidogyne* was ranked first of the ten most important genera of plant-parasitic nematodes (Sasser and Freckmann, 1987).

Control of root-knot nematodes has traditionally been achieved by use of fumigant and non-fumigant chemical nematicides. These are, however, expensive and unaffordable to most small-scale farmers. It is acknowledged that chemical nematicides disturb soil biodiversity causing an imbalance between beneficial and deleterious organisms (Yeates, 1996). Currently, some of the popular nematicides are being banned from the global market due to environmental and health concerns. Use of crop rotation and fallowing as

management practices can be support solutions to reduce damage by root-knot nematodes which have a large host range. Field application of organic soil amendments to control root-knot nematodes is hindered by enormous amounts of material required for effective treatment, this method is largely restricted to systems where only small portions require nematode control.

Occurrence of soils that suppress build-up of nematode populations in fields continuously planted with susceptible crops has been reported (Stirling *et al.,* 1979; Hidalgo-Diaz *et al.*, 2000). Natural regulation of plant parasitic nematodes in these soils is considered to result from a wide range of antagonistic microorganisms including nematode destroying fungi, obligate bacterial parasites, rhizobacteria, and fungal endophytes (Stirling, 1991; Sikora, 1991; Sikora, 1992). Control of root-knot nematodes using biological agents such as nematophagous fungi *Verticillium chlamydosporium* Goddard [synonym: *Pochonia chlamydosporia* (Goddard) Zare & Gams] and *Arthrobotrys oligospora* (Cooke & Godfrey) Drechsler, alone or in combination with other control methods in integrated pest management programs is thought to be a

suitable alternative to conventional methods of nematode control (Kerry, 2001). However, information about the activity of these biocontrol agents, their ecological requirements and potential risks associated with their use is needed. The susceptibility of the host to the nematodes and their rate of development and multiplication; rate of growth, sporulation, virulence and rhizosphere competence of the fungal agent are factors which are likely to influence the impact of the nematode antagonist in suppressing nematode populations (Kerry and Bourne, 1996).

Most studies on the activity of nematophagous fungi are terminated after only one or two generations. Information is needed on the effect of the antagonistic fungi on nematodes between successive crops. The objective of this study was to assess the efficacy of isolates of nematode destroying fungi *V. chlamydosporium* and *A. oligospora* in biological control of *Meloidogyne incognita* (Kof & White) Chirtwood, on celery and tomato and to determine the persistence of the biocontrol agents in the rhizosphere of these crops.

Materials and Methods

A greenhouse experiment was conducted between March and August 2001 to determine the efficacy of nematode destroying fungi in control of root-knot nematodes on celery and tomato.

One isolate of *V. chlamydosporium* (Vc-10) was obtained from Entomology and Nematology Department, Rothermsted Experimental Station, Harpenden,UK. It was originally isolated from eggs of *M. incognita* and it previously controlled *M. incognita, Meloidogyne. hapla, and Meloidogyne. arenaria* (Irving and Kerry, 1986). A second isolate (Vc-2 M), another of *V. chlamydosporium* was provided by the Laboratory for Phytopathology and Plant Protection, Katholieke Universiteit Leuven, Belgium. Isolate Vc-2M was originally isolated from cysts of beet cyst nematodes (*Heterodera schachtii)* by Coosemans and Hojat, (1995). De Ceuster, a commercial company, provided the isolate of *A. oligospora.*

Medium for re-isolation of *V. chlamydosporium* from the soil was prepared using the procedure described by Kerry et al. (1990). The ingredients included: 17 g corn meal agar, 17 mg NaCl, 75 mg rose Bengal, 37.5 mg each of carbendazim and thiabendazole, 3ml of Triton X 100, 50 mg each of streptomycin sulphate, auromycin and chloramphenicol mixed thoroghouly in 1 litre of distilled water in an Erlenmeyer flask and autoclaved at 121°C for 15 min. The medium was allowed to cool and dispensed into 9 cm diamater petri-dishes in a laminar hood.

M. incognita was maintained on tomato (*Lycopersicon esculentum*) variety Moneymaker in a greenhouse. Nematode inoculum production was done using the method described by Hussey & Baker, (1973). Galled tomato roots were gently washed free of soil and immersed in 200mls of 13% NaOCl solution in a conical flask. The roots were shaken vigorously in NaOCl solution for 4 min and passed through a 200 mesh sieve nested in a 500 mesh sieve to collect the freed nematode eggs. The eggs were collected on 50 µm sieve and rinsed in a stream of tap water to remove residual NaOCl. The eggs were placed on paper towels supported by a Baermann funnel and incubated in water at 24°C. After 14 days, second stage (J2) infective juveniles were collected by opening the tap of the funnel into a vessel and counted using a nematode counting slide. The potting soil (pH 6.5 and containing 20% organic matter) was infested with J2 at the rate of 100 juveniles and eggs/100g soil.

Chlamydospores of *V. chlamydosporium* isolates were produced according to the procedure described by Bourne et al. (1994). Two grams of milled barley was washed through a 53µm sieve. The washed barley was mixed with an equal volume of coarse sand. 100 g quantities of the mixture were put in 250ml conical flasks and autoclaved at 121°C for 15 min. The mixture was inoculated with 5 plugs of 5mm diameter from 3 weeks old cultures of *V. chlamydosporium* isolates on corn meal agar (CMA), these were incubated at 25°C for 21 days. The colonized medium was washed onto the 250 µm sieve over 75 µm sieve onto 50 µm sieve. The chlamydospores and some hyphal fragments were collected on a sieve with 10µm openings. The suspension for each strain was added to 3kg of sterile seeds of millet (*Panicum milioceum* L.) at the rate of 7.0×10^6 chlamydospores/g seed for Vc-2M, and 2.2×10^6 chlamydospores/g seed for Vc-10.

Assessment of isolates of *V. chlamydosporium* and *A. oligospora* for control of root-knot nematodes and their survival in the plant rhizosphere was done using celery and tomato in the greenhouse. A similar experimental

design was adopted for both crops. There were 5 treatments in each as follows:

- Soil treated with Vc-2M and infested with infective juveniles of *M. incognita*
- Soil treated with Vc-10 and infested with infective juveniles of *M. incognita*
- Soil treated with a preparation of *A. oligospora* and infested with infective juveniles of *M. incognita*
- Soil infested with infective juveniles of *M. incognita* only
- Soil treated with sterile water only

Treatment of the soil with biocontrol agents and introduction of the nematode inoculum was done prior to planting with four weeks old celery (*Apium graveolus* L.) seedlings. The rates of application of the biocontrol agents were 3×10^5 chlamydospores/g soil for Vc-2M, 10^5 chlamydospores/g soil for Vc-10 according to Bourne *et al*., 1994 and Coosemans, 1991. A test isolate of *A. oligospora* was applied at the rate of 70 ml/70 kg soil (concentration 1×10^5 cfu/ml). The treatments were replicated four times, and arranged in a completely randomized design. Four plants were maintained per pot in the greenhouse at 20°C and watered regularly as required. The experiment was terminated eight weeks after planting.

After data collection, roots were retained and incorporated appropriately in to soil of respective treatments. The soil was turned completely and directly sown with seeds of tomato, cultivar Moneymaker seeds. The pots were watered and four plants maintained in the greenhouse at 20°C for a further 8 weeks. Data on egg mass index, galling index, number of juveniles in the soil, number of chlamydospores/g of soil, percentage of infected egg/ egg masses, and fresh shoot/root weight were collected.

Damage to the roots by the nematodes was estimated using a galling index according to the scale of 1-9 (Mullin *et al*., 1991). (Where1=no galling and 9=76-100% of root system galled or completely destroyed by nematodes and opportunistic fungi). The roots were treated with Phloxine-B organic stain for easy observation of egg masses on the roots.

A scale of 0-5, Where $0 = 0$ and $5 =$ over 100 egg masses per root system (Taylor and Sasser, 1978) was used to assess the egg masses on the root system of the plants from each pot. The numbers of chlamydospores/g of soil obtained from celery and tomato rhizospheres were determined using the method described by de Leij & Kerry, 1991. Serial dilutions were made with *V. chlamydosporium* being re-isolated on the semi-selective medium developed by Kerry *et al*. (1990), while *A. oligospora* was re-isolated on potato dextrose agar. Cultures were incubated at 18°C and colonies that emerged after 10 days were counted using a haemocytometer.

Extraction of nematodes was done using Modified Baermann's method (Gooris and D'Herde, 1972). The isolated juveniles were counted using a counting slide. Reproduction of *M. incognita* was estimated using the ratio of final population of hatched infective juveniles to the initial population infested in the soil (both populations expressed as number of juveniles/100g soil).

To determine the percentage infection of egg masses, twenty-five randomly picked egg masses were plated on the semi-selective medium in four replicates and incubated at 18°C for ten days. The eggs were examined for growth of *V. chlamydosporium* with a stereomicroscope at ×60 magnification

Results
All data were subjected to a one-way analysis of variance using SPSS for Windows Release 9.0, 1998 computer program and the means were compared using LSD and Duncan's multiple-range test where there were significant differences..

Root-knot nematode infestation did not result in significant ($p \leq 0.05$) reduction in fresh shoot weight of celery. However, treatments differed significantly ($p \leq 0.05$) in fresh root weight, degree of root galling and the number of hatched juveniles of *M. incognita* in the soil. Vc-2M and Vc-10 reduced reproduction of *M. incognita* and its damage to celery. Plants from pots treated with Vc-2M had a mean galling index of 5.7±0.2 and a nematode reproductive factor of 1.7±0.04 whereas those from pots with Vc-10 had a galling index of 5.1±0.4 and a nematode reproductive factor of 1..2±0.1. Treatments with *A. oligospora* did not differ significantly ($p \leq 0.05$) in root galling and rate of reproduction of the nematode from the untreated check inoculated with the nematode alone (Table 1).

Table 1: Mean fresh root, shoot weight; galling index as damage parameters and reproductive factor of *Meloidogyne incognita* on celery treated with *V. chlamydosporium* and *A. oligospora,* eight weeks after planting the seedlings.

	Fresh shoot Weight. (g)	STD	Fresh root Weight. (g)	STD	Galling Index**	STD	Rf.	STD
Vc-2M	51.1a	27.4	8.2a	10.8	5.7a	1.3	1.7a	0.1
Vc-10	54.1ab	27.3	12.0ab	8.3	5.1a	2.1	1.2a	0.1
Abs. Control	52.3ab	27.8	15.9b	10.8	1.0b	0.0	0.0	0.0
Arthrobotrys	39.9b	16.1	20.8c	13.1	7.4c	1.0	3.2b	0.2
Nem. Control	40.9ab	17.1	27.7d	12.6	8.2c	0.7	3.7b	0.1
SED	2.0		1.1		0.2		0.3	

Means in each column followed by the same letter are not significantly different at p≤0.05 according to Duncan's multiple range test.
 ** Galling Severity Index rating scale of 1-9 where 1= no galling, 2= <5%, 3= 6-10%, 4= 11-18%, 5= 19-25%, 6= 26-50%, 7= 51-65%, 8= 66-75% and 9= 76-100% of root system with galling (Mullin *et al*., 1991). Rf is reproductive factor of the nematode on host plant; SED standard error difference; STD standard deviation

There were significant differences (p≤0.05) between and within treatments in the number of egg masses per root system, root galling index and the percentage of infected eggs per egg mass. The two isolates of V. chlamydosporium (Vc-2M and Vc-10) significantly (p≤0.05) controlled nematode damage to tomato. Plants treated with Vc-10 had the lowest galling index (2.3) and egg mass index of 2.6 while those treated with Vc-2M had galling and egg mass index of 2.7 (Table 2). Application of A. oligospora did not give any significant control (p≤0.05). Both strains of V. chlamydosporium survived in the rhizosphere of tomato plants and were found parasitizing eggs of M. incognita 16 weeks after application to the soil; Vc-10 had the highest percent infectivity (66.1%). Vc-2M had infectivity of 53.8%. Egg infection in the untreated check and in soil treated with A. oligospora was below 10% (Table 2).

There were significant differences (p≤0.05) between treatments in the number of chlamydospores/g soil in plant rhizosphere and percentage of infected egg masses in both tomato and celery experiments. Vc-10 had the highest number of cfu (1.35×10^5 cfu/g soil) in tomato rhizosphere and (1.2×10^4 cfu/g soil) in celery rhizosphere. The final soil concentration of V. chlamydosporium did not vary appreciably from the initial soil concentration (10^5 cfu/g soil, Vc-10 and 3×10^5 cfu/g soil, Vc-2M) sixteen weeks after soil infestation (Table 3). Hyphae of both strains of V. chlamydosporium proliferated on the infected egg masses and penetrated them to cause massive destruction to the eggs contained in the gelatinous matrix.

Table 2: Galling severity index (GSI), egg mass index (EMI) and % infectivity of *Meloidogyne incognita* eggs by *V. chlamydosporium* on tomato (Values are Means ± SD, N=25).

Treatment	EMI*	Galling Index**	***% egg Infection
Vc-2M	2.7 ± 0.95[ab]	2.7± 0.95a	53.8± 4.6a
Vc-10	2.6 ± 0.50[a]	2.2± 0.50a	66.2± 3.1a
Arthrobotrys	3.3 ± 0.50bc	6.0± 0.81b	8.7± 1.7b
Nem. Cont.	3.8 ± 0.50c	6.3± 2.10b	7.6± 2.4b
Abs. Cont.	0.0 ± 0.00	1.0± 0.00	0.0± 0.0
SED	0.3	0.5	6.8

Means in each column followed by the same letter are not significantly different at p≤0.05 according to Duncan's multiple-range test.

STD standard deviation; SED standard error difference. *Egg Mass Index (EMI) rating scale of 1-5 where, 0=0, 1=1-2, 2=3-10, 3=31-100, 5>100 egg masses per root system (Taylor and Sasser, 1978). **Galling Severity Index (GSI) rating scale of 1-9 (Mullin *et al*., 1991). ***Percentage of eggs in observed egg masses that were infected by *V. chlamydosporium*.

Table 3: Percent egg mass colonization and fungal survival in the rhizosphere of plants.

	% infected egg-masses on tomato	STD	10^4 cfu/100g soil tomato	10^3 cfu/100 g soil celery	STD
Vc-2M	80.5a	3.1	9.25	4	1.7
Vc-10	98.5a	1.3	13.5	12	1.29
Nem. Cont.	4.5b	1.3	0.0	0.0	0.0
Arthrobotrys	4.3b	1.7	0.01	-	
SED	11.1		0.85	0.64	

Means in the same column followed by the same letter are not significantly different (p≤0.05) according to Duncan's multiple-range test; STD standard deviation; SED standard error difference

Table 4: Correlation between damage to tomato plant by root-knot nematode (severity galling index) and survival and/or pathogenicity of *V. chlamydosporium* (cfu and egg mass colonization).

		Galling Severity Index.	CFU.	Egg mass Colonization.
Galling Severity Index.	Pearson correlation	1.00	-0.63*	-0.87**
	Sig. (2-tailed)	0.00	0.03	0.00
Colony forming Units.	Pearson correlation	-0.63*	1.00	0.79**
	Sig. (2-tailed)	0.28	0.00	0.00
Egg mass Colonization.	Pearson correlation	-0.87**	0.79**	1.00
	Sig. (2-tailed)	0.00	0.00	0.00

• Correlation is significant at 0.05 level (2-tailed), ** Correlation is significant at 0.01 level (2-tailed).

In this study, percent egg mass infection by the fungus most influenced the damage to plants by root-knot nematode, there was significant negative correlation (–0.87) between galling severity index and egg-mass colonization. Damage to the crops by the nematode decreased with increase in the percentage of its infected eggs (Table 4).

Discussion and Conclusion
Fungal proliferation in the host plant rhizosphere is a prerequisite for suppression of root infecting nematodes (de Leij & Kerry, 1991); survival in plant rhizosphere of the biological control agents varied significantly; an isolate of *A. oligospora* for example persisted only up to the end of celery crop (at the density of 100cfu/100g soil) and could not be re-isolated from the rhizosphere of tomato. Plants treated with this agent had the highest galling index and highest number of free-living infective juveniles in the soil. The predaceous activity of nematode trapping fungi is dependent on the inoculum density (Cooke, 1963). Lack of nematode control by *A. oligospora* in this experiment may have been due to insufficient density or due to antagonistic activity of indigenous soil microfauna. Successful control of the nematode by trapping fungi also depends on

the efficiency of trap formation while the infective juveniles are still migrating freely in the soil. In this experiment, the infective juveniles of *M. incognita* may have penetrated and entered the root tissues before *A. oligospora* ensnared them. Poinar and Hess (1988); Simon *et al.* (2003) made similar observations.
There was no significant variation between isolates of the egg-parasitic fungus *V. chlamydosporium.* Both isolates survived in the rhizoplanes of celery and tomato for at least sixteen weeks. Eight weeks after planting celery, the soil concentration of Vc-10 was 1.35×10^5 cfu/g soil and 9.25×10^4 cfu/g soil for Vc-2M. These fungi gave 62.2% and 53% infection of nematode eggs respectively. Observation of infected egg masses under a camera-mounted microscope revealed a massive invasion of the eggs by the fungus, after destruction of the egg mass contents the fungus formed survival structures (chlamydospores). These isolates were typical egg-parasites (de Leij & Kerry, 1991) and therefore did not prevent initial nematode invasion, they controlled later generations of the nematode and therefore control was more on tomato than celery. Both isolates of *V. chlamydosporium* significantly reduced the reproduction rate of the nematode. The

concentrations of the strains of *V. chlamydosporium* remained fairly constant because of several possible reasons: peat soil has an upper limit to it's carrying capacity of about 10^5 cfu/g soil (de Leij *et al*, 1993); it may have also been due to competition for substrate.

Both fungal antagonists on one hand, and their nematode host on the other hand suffered an interruption during a shift to the next crop in the experiment. It takes a long time for antagonists to establish equilibrium with their nematode host so as to suppress its population to sub threshold levels (Stirling, 1991).

Isolate Vc-10 of *V. chlamydosporium* was the most virulent of the three tested pathogens of root-knot nematode. It infected 98% of the nematode egg masses and had an average egg infection of 62% in these egg masses. It caused a significant reduction of the hatched juveniles in the later nematode generations leading to reduced damage to the treated plants; nematode control was most evident on tomato. Isolate Vc-2M also caused a significant reduction of nematode damage to the tomato but was not as effective as isolate Vc-10.

Both isolates of *V. chlamydosporium* (Vc-10 & Vc-2M) survived in the rhizospheres of the two nematode infected crops and could be re-isolated at least sixteen weeks after they were introduced into the soil. Their final soil densities were 1.35×10^5 cfu/g soil and 9.25×10^4 cfu/g soil respectively. *A. oligospora* was however only present in the rhizosphere soil up to the end of the first crop (at 100cfu/100g soil).

References

Bourne JM, Kerry BR and De Leij FAAM 1994 Methods for the study of *Verticillium chlamydosporium* in the rhizosphere. Journal of Nematology 26 (4S): 587-591.

Bridge J 1987 Control strategies in subsistence agriculture In Brown, RH and Kerry, BR eds Principles and Practice of Nematode Control in Crops, pp 389-420 Academic Press, Marrickville NSW, Australia

Cooke RC 1963 The predaceous activity of the nematode trapping fungi added to the soil. Annals of Applied Biology 51:295-299.

Coosemans J 1991 Methods for introducing *Verticillium chlamydosporium* into soil In: *Methods for Studying Nematophagous Fungi.* Pp 39-46 Eds B R Kerry & D H Crump Vol 14/2, 10BC WPRS Bulletin

Coosemans, J and JAAA Hojat 1995 Antagonistic fungi of sugar beet cyst nematodes in Iran Proceedings of the 12th Iranian Plant Prot Congress Karaj, Sept, 7-12.

De Leij FAAM, Kerry BR and Dennehy JA 1993 *Verticillium chlamydosporium* as a biological control agent for *Meloidogyne incognita* and *M*

hapla in pot and microplot tests *Nematologica,* 39: 115-126.

De Leij FAAM and Kerry KR 1991 The nematophugus fungus *Verticillium chlamydosporium* as a potential biological agent for *Meloidogyne arenaria. Reiview of Nematology* 14:587-1991

De Leij F A A M, B R Kerry, and J A Dennehy 1992: The effect of fungal application rate and nematode density on the effectiveness of *Verticillium chlamydosporium* as a biological control agent for *M incognita. Nematologica,* 38: 112-122

Gorris, J and C J D'Herde 1972 A method for quarantine extraction of eggs and second stage juveniles of *Meloidogyne* spp from soil Ghent State Agric Res center

Hidalgo-Diaz L, Bourne JM, Kerry BR, and Rodriguez MG 2000 Nematophagous *Verticillium* spp in soils infested with Meloidogyne spp in Cuba: isolation and screening *Internat J Pest Manag* 46:277-284

Hussey RS and Barker KK 1973 A comparison of methods of collecting inocula for *Meloidogyne* spp including a new technique. Plant Diseases Reports 57:1025-1028.

Irving F and Kerry BR 1986 Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard II Factors affecting parasitism of cyst nematode eggs. *Nematologica,* 32: 474-485

Kerry BR and Bourne JM 1996 The importance of rhizosphere interactions in the biological control of plant parasitic nematodes a case study using *V Chlamydosporium.* Pesticide Science 47, 69-75

Kerry BR 1990 An assessment of the progress towards biological control of plant parasitic nematodes Journal of Nematology 22:621-631

Kerry BR 2001 Exploitation of the nematophagous fungus *Verticillium chlamydosporium* Goddard for bilogical control of root knot nematodes (Meloidogyne spp), in *Fungi as biocontrol agents*, ed by Butt TM, Jackson C and Magan N, CAB International, Wallingford, UK, pp 155-167

Mullin BA, Abawi GGS, Pastor-Corrales MA and Kornegay JL 1991: Reactions of selected bean pureline accessories to *Meloidogyne* species. Plant Dieases 76:1212-1216

Poinar GO and Hess R 1988 In: Diseases of Nematodes, Vol 1 (GO Poinar and HB Jans-Sons, eds) pp; 103-131 CRC Press, Boca Raton, Florida

Poinar GO and Hess, R 1988 In: Diseases of Nematodes Vol 1 (GO Poinar and HB Hansson, eds) pp 103-131 CRC Press, Boca Raton, Florida

Sasser JN and Freckmann JN 1987 A world perspective of nematology: The role of society In Veech , JA and Dickson, DW eds Vistas on Nematologists, Pp7-14

Sikora RA 1991 The concept of using plant health promoting rhizobacteria for the biological control of plant nematodes Proc 2nd Int Workshop Plant Growth-promoting Rhizobacteria Interlaken, Switzerland WRRS Bull XIV/ 8:3-10

Sikora RA 1992 Management of the antagonistic potential in the agricultural ecosystems for the biological control of the plant parasitic nematodes *Annual Review Phyotopathology* 30:245-270

Simon DA, Hidalgo-Diaz L, Kalisz H, Mauchline, TH, Hirsch PR and Kerry BR 2003 Development of a new management strategy fpr the control of root-knot nematodes (*Meloidogyne* spp) in organic vegetable production. Pest Management Science 59:183-189.

SPSS for Windows Release 900, SPSS Inc 1989-1999

Stirling GR 1991 Biological control of plant parasitic nematodes Wallingford, UK, CABI, pp 282.

Journal of Tropical Microbiology and Biotechnology

Instructions for Authors (Last Updated December 2007)

STATEMENT OF SCOPE

The Journal of Tropical Microbiology and Biotechnology (formerly Journal of Tropical Microbiology) publishes papers and critical reviews in Medical Microbiology, Plant pathology, Public Health, Industrial Microbiology, Biotechnology, Environmental Microbiology, Microbial Pathogenesis, Food Microbiology, Microbial Physiology, Biochemistry of micro-organisms, Microbial Genetics, Molecular Biology, Bacteriology, Virology, Mycology, and Microbial Systematics. Both full length and short papers reporting original research making a significant contribution to microbiology and Biotechnology will be considered for publication. Letters to the Editor are welcome when these comment on matters relevant to Microbiology and Biotechnology. Reviews can be published on request from the Editor. The primary criterion for publication is new insight that is of broad interest to Microbiologists and Biotechnologists.

MANUSCRIPT SUBMISSION

Before initiating the submission process, these Instructions for Authors should be read in full (available at http://www.ajol.info). If an author does not have access to the Internet, contact the Editor in-chief, Prof. Jesse Machuka machuka@mitsuminet.org or the Assistant editor-in-chief Prof. Hamadi Boga hamadiboga@yahoo.com

Authors should submit manuscripts by e-mail to the Editor–in–chief and copy it to the Assistant Editor in Chief. A manuscript file in Microsoft Word or Rich Text Format is required.

Cover Letter. A cover letter must be submitted along with the manuscript, explaining why the authors believe it meets the journal's scope and noting that the manuscript has not been submitted for publication elsewhere and that all co-authors have agreed to its submission.. Any closely related papers that are in press or that have been submitted elsewhere should be noted in the cover letter, cited in the submitted manuscript.

MANUSCRIPT PREPARATION AND ORGANIZATION

Manuscripts should follow *The Journal of Tropical Microbiology and Biotechnology* style, be written in concise and grammatically correct English. Consult a current issue of *The Journal of Tropical Microbiology and Biotechnology* for guidance on format, organization, and preparation of figures, legends, tables, and references. In general, there are no limits to the length of manuscripts published in *The Journal of Tropical Microbiology and Biotechnology*; however, papers of more than 10 journal pages will be published only if the editor judges that the content is sufficiently novel to warrant a long paper. Original manuscripts must be prepared in 12 point type using the Times Roman font. Use settings for standard A4 paper. Figures should be digitally prepared (see below).

Organize manuscripts in the following order: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Figure *Legends*, Tables, and References. **Note that when submitting your manuscript the References must be placed at the end of your document file**. Tables and legends should be included as part of your manuscript file if possible.

Title Page. The title page should include the authors' full names and affiliations, a running title of 40 characters or less (in addition to the full paper title), and the telephone and fax numbers and e-mail address of the *corresponding* author.

Manuscript Title. The full manuscript title should be succinct (approximately 90 characters) and informative. The title should include sufficient detail for indexing but be general enough to be clear to the broad readership of the *journal*. Toward that end, abbreviations and acronyms, including those for gene and protein names, should be avoided in titles or the general nature of the abbreviated entity should be clear. The journal encourages active titles over merely descriptive ones whenever possible. The title must mention the subject organism (or general group in the case of comparative works). Common names are allowed for major model systems (maize, rice, yeast); Latin names should be used for all organisms that have no standard (widely accepted) common name.

Abstract. The abstract should stand on its own with no reference to the text. It should contain approximately 200 words and must summarize the *questions* being addressed, the approach taken, the major findings, and the significance of the results. It should be concise, complete, and clearly communicate the importance of the work for a broad audience.

Introduction, Materials and Methods, Results and Discussion. Manuscripts should be written with sufficient clarity and conciseness to be understood by a wide audience of plant biologists, not only

specialists. The use of abbreviations and jargon should be avoided, and terms that are not widely known should be explained clearly at first use. The Introduction should provide the necessary background information for the average reader; it should be both complete and concise. Previous publications that form a basis for the work presented must be cited. Citation of reviews is not a substitute for citing primary research articles. Citation of recent research articles is not a substitute for citing original discoveries. An author's own work should not be cited preferentially over equally relevant work of others. Authors' previously published data that are presented along with new data must be identified clearly and cited appropriately; duplicate publication of data (including data previously published as supplemental material) is not allowed without citation. Methods must be described completely enough that other laboratories can replicate results and verify claims. Generally, standard procedures should be referenced, though significant variations should be described. Appropriate experimental design and statistical methods should be applied and described wherever necessary for proper interpretation of data and verification of claims. All novel materials and the procedures to prepare them should be described in sufficient detail to allow their reproduction (e.g., DNA constructs, genetic stocks, enzyme preparations, and analytical software). Authors must provide the city and state, or country, of manufacturers in the Methods section. The Results and Discussion can be subdivided if subheadings give the manuscript more clarity. The Discussion should not repeat the Results; instead, the Discussion should explore the implications of the Results, citing relevant published research, and should also be as concise as possible. A Conclusions section is generally not permitted.

Line Numbering. The lines in the manuscript should be numbered on the left margin. Line numbering can easily be done in word documents by going to File/Page setup/page layout/line numbering.

Nomenclature and terminology. *The Journal of Tropical Microbiology and Biotechnology* requires that all nomenclature, including gene names and symbols, conform to nomenclature conventions adopted by the scientific community and that all genetic terminology be used in a scientifically accurate manner.

Organisms. In the Abstract, text, and Methods, organisms should be referred to by their common name at first use (if a standard common name applies), and the Latin name should be given in parentheses. Subsequent references to organisms can be either Latin or common names but should be consistent throughout the manuscript.

Authors are responsible for determining that all nomenclature conforms to accepted community standards prior to submission.

Accession numbers. Accession numbers should be provided as the last paragraph of Methods (just before Acknowledgements) with the heading "Accession Numbers" for any data or materials available in a public repository. If an accession number has not been assigned at the time of submission, please use Xs as placeholders to be updated later. In general, accession numbers should be provided only in the Methods, not elsewhere in the manuscript, unless necessary for clarity.

Figure Legends. Provide a short title for each figure. Figure legends should be concise and should not repeat information presented in the text. Figure panels that are designated with capital letters should have specific subtitles in the legend and should be described separately and completely. Do not describe methods in figure legends unless they are necessary to interpret the results conveyed by the figure. Define in the legend all symbols and abbreviations that are used in the figure. Accession numbers should not be included in figure legends.

Tables. Tables should be submitted embedded within the text file just before the references. Prepare tables using Word's table feature. If not using Word's table feature, tables can be prepared using a single tab between columns. Number tables consecutively as they are first mentioned in the text. Provide a concise title for each table, and label each column with an unambiguous heading. If footnotes are needed for clarity, designate them with lowercase lettersin the order in which they are referenced in the table. Table titles and footnotes should be placed as regular text outside the table body. Each table may include a short general description before the footnotes.

References. Cite references in the text by name and date of publication and not by number. List only articles that are published or in press. References should contain complete titles and inclusive page numbers. Authors are expected to proofread every citation in their reference list against the PDF or photocopy of the cited work so that the reference list is accurate with respect to spellings, symbols, italics, subscripts/superscripts, and accents. Cite in the text all unpublished results, including personal communications and submitted manuscripts (for example, R. Goldberg and K. Jofuku, unpublished results). Citations for web sites (other than for primary literature) should be handled parenthetically in the text and not included in the reference list. Authors should test all URLs and links.

All cited publications should be read by the authors. Reference to specific results must be to original publications, not to later publications or reviews.

Figures. Figures should be provided seperately as TIFF, JPEG, PPT or PDF files and should not be included in the word docment, to avoid large documets. Number figures consecutively according to the order in which they are called out in the text. Figures should be unambiguous and as conceptual as possible and should provide enough information so that the reader can understand them without significant input from the text. Whenever possible, position panels vertically for one-column reproduction in the journal. For the best possible reproduction of gel blots, submit combination figures in which the labels and photographs or autoradiographs are composite images. Format the width of sequence data in the paper to one column.

Use the same typefaces for all figures. For those figures that contain more than one panel, designate the panels with capital letters (no parentheses and no periods following letters) in the upper left-hand corner of each panel. Wording in figures must match the rest of the manuscript for capitalization, italics, and use of symbols. If labels contain typographic errors or inconsistencies, the author will be asked to upload corrected figures.

Figure format. We recommend that figures be created using Adobe Photoshop or Illustrator. If you use Photoshop or similar software, send tiff files at full size and delete any blank space around the edges of each figure. Resolution of at least 600 dpi is needed for most figures; 1000 dpi is needed for any figures that are made entirely of black lines and text (e.g., line graphs). If you use Illustrator or similar software, send eps files. Type should be set to "create outlines" or "convert to paths," or fonts should be embedded when saving the file. If you use PowerPoint, send the original PowerPoint files. Use only basic PowerPoint fonts, do not draw lines that are less than .25 points thick, and use shaded or colored fills instead of "pattern" fills. Images imported into PowerPoint should have at least 600 dpi resolution.

All parts of a figure must fit on a single printed page and if figures are too large each figure should be sent in its own file.

Cover Submissions. Each cover of *The Journal of Tropical Microbiology and Biotechnology* will feature an image from one of the articles published in that issue. Authors who wish to have an image considered for the cover should upload the image as a supplemental file and indicate on the checklist provided at acceptance that a cover candidate has been submitted. If an author does not hold the copyright for a submitted image, they are responsible for obtaining the necessary permission to use the image in *The Journal of Tropical Microbiology and Biotechnology*.

Extras

♦ When giving the page numbers e.g. 12-15 use the longer dash 12–15, which can be done by pressing the command (control+, minus).
♦ The simple brackets (Okemo 1992) should be used when enclosing one set of information as shown above. However in case more than one set of brackets are needed then let it be done as follows e.g. [2.5 mg dry wt (mol of substrate)$^{-1}$]
♦ The multiplication symbol ($\times$) should be used and not (x or X).

PEER REVIEW

Members of the editorial board will evaluate all manuscripts upon submission to determine whether they are appropriate for evaluation by expert outside reviewers. Editors are permitted to use any reviewer reasonably believed to be an appropriate scientific expert, except reviewers who would be excluded by KSM's conflict of interest policy. Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors within 4 weeks whenever possible. If revision is requested, the editorial board will evaluate revised manuscripts and determine whether outside review is required. The board normally will consider only one revised manuscript, and this manuscript must be submitted within 1 month unless an extension is granted. In the case that extensive revision including additional experimentation is required, journal policy is to decline the manuscript, but editors may choose to encourage resubmission. Resubmissions are subject to the full review process. It is the goal of the journal to publish manuscripts within 2 months after submission, but this can only be achieved if the original submission meets all journal requirements.

MANUSCRIPTACCEPTANCE

Authors of accepted manuscripts will be provided with a checklist for preparing their final submission. Accepted manuscripts are copyedited for grammar and journal style before proof is generated. Any subsequent final corrections to the paper will appear in the final published version for both *The Journal*

of Tropical Microbiology and Biotechnology Online and *The Journal of Tropical Microbiology and Biotechnology* (paper) versions.

PROOFS

Electronic page proofs will be delivered to the corresponding author. The author will have access to a PDF file, which will contain PDF pages (with figures, images and tables). Page proofs are considered to be the final version of the manuscript. **With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.** Notes added in proof will be sent to the co-editor assigned to the manuscript prior to publication and will be reviewed for appropriate content and wording. Authors will receive proofs approximately 3 to 4 weeks after final acceptance of the manuscript. Because of the tight publication schedule, authors must relay all additions and corrections to the printer by e-mail or by overnight mail within 24 hours of receipt of the proofs. Failure to act promptly to approve the page proofs may delay publication of the manuscript. For electronic correction of manuscripts, please use the free text tool to mark your PDF proof.

FEES AND CHARGES

Members of the Kenya Society of Microbiology will be charged a fee of KSh 5000 for the first five pages and 200 KSh for any extra page. Corresponding authors who are non-KSM members will be charged US$ 200 per manuscript. Payments should be made to Kenya Society of Microbiology, Kenyatta Avenue Branch, Account Number 0152012639200, Swift code: SCBLKenX

FEATURE ARTICLES

The front section of the journal features occasional editorials, In this issue articles, Current Perspectives Essays, Historical Perspectives Essays, reviews, and letters/commentaries. Prospective authors may inquire about contributing an article to one of these features by contacting Prof. Jesse Machuka (machuka@mitsuminet.com) or Dr. Hamadi Boga, (hamadiboga@yahoo.com)

Corrections and Retractions. If necessary, corrections of significant errors in published articles will be published in a later issue of the journal. Within 2 months after publication, authors are requested to bring any errors to the attention of the managing editor. A correction published in the print journal will also be published as a correction in the online journal and will be linked to the original article. Articles may be retracted by their authors, academic or institutional sponsor, editor, or publisher because of pervasive error or unsubstantiated or irreproducible data. A correction or retraction, so labeled, will appear in a prominent section of the journal, will be listed in the contents page, and will include the title of the original article. The text of a retraction will explain why the article is being retracted and will include a bibliographic reference to it.

Contacts

Prof. Jesse Machuka
Department of Biochemistry
Kenyatta University
P.O. Box 43844-00100
Nairobi, Kenya
machuka@mitsuminet.com   or
machuka.jesse@ku.ac.ke
Fax: 254-2-811575
Tel. 2542810245
Cell: 254-720-288526

Kenya Society of Microbiology,
P.O. Box 10696-00100 Nairobi,
Kenya
Fax: 254-2-811575
Tel. 2542810245

Prof Hamadi Boga
JKUAT, P.O. Box 62000-00200, Nairobi, Kenya
hamadiboga@yahoo.com or
hboga@fsc.jkuat.ac.ke
Mobile: 00254733926733
Office: 002546752711
Fax: 002546752164